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STUDIES ON MOTILITY IN *RHODOMICROBIUM VANNIELII*

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Thesis submitted for the degree of Doctor of Philosophy,  
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## SUMMARY

The change from swarmer cell to non-motile reproductive cell was examined as a landmark event in differentiation of the purple non-sulphur bacterium, *Rhodospirillum rubrum*, using synchronised swarmer cell populations.

It was found that shed flagella from *Rm. rubrum* consisted of 3 proteins: flagellin,  $M_r$  34k, hook protein,  $M_r$  36k and a polypeptide of  $M_r$  37k, possibly rod protein. No methyl-accepting chemotaxis proteins were detected in *Rm. rubrum*.

Radioimmuno-precipitation was used to determine the period of flagellin synthesis during differentiation. Synthesis of flagellin was switched off in *Rm. rubrum* swarmer cells after 1-2 hours anaerobic incubation in the light. If swarmer cells were held anaerobically in the dark, flagellin synthesis continued for at least 6 hours. Thus, swarmer cells, which have a dispersal role in nature, detect whether environmental conditions are conducive to completion of the cell cycle, and regulate their gene expression accordingly. This contrasts with conclusions drawn from work with the non-photosynthetic aquatic bacterium, *Caulobacter crescentus*, whose cell cycle also includes a swarmer cell to non-motile reproductive cell transition.

In order to study control of flagellin expression further, cloning of the gene(s) was attempted. Heterologous Southern hybridisations between restricted *Rm. rubrum* chromosomal DNA and the cloned 29k flagellin gene from *C. crescentus* showed that 55-60% homology existed between the two. This was judged to be too weak a signal to allow cloning of *Rm. rubrum* flagellin genes by hybridisation. Antibodies raised against *Rm. rubrum* flagellin and hook protein did cross react with *C. crescentus* flagellins and hook protein.

Screening a library of *EcoRI* digested *Rm. rubrum* chromosomal DNA in the vector  $\lambda$ gt11 with anti-flagella antiserum indicated that *Rm. rubrum* flagellin and hook protein were not expressed in *E. coli* from DNA cut in such a way.

Eleven Tn5-induced motility mutants of *Rm. rubrum* were isolated (3 of which were chemotaxis deficient), and these should enable the cloning of genes for motility from this organism in the future.



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## DECLARATION

I declare that this thesis has been composed by myself from the results of research conducted under the supervision of Dr. C. S. Dow and has not been used in any previous application for a degree. All sources of information have been acknowledged by means of a reference.

*Julie Macdonald*

J. Macdonald

## ABBREVIATIONS

Ap	ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
cAMP	3',5'-cyclic adenosine monophosphate
CI	Curie
Cm	chloramphenicol
c.p.m.	counts per minute
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EAN	envelope associated nucleoid
EDTA	diaminoethanetetra-acetic acid
e.m.	electron microscope
FCCP	carbonylcyanide-p-trifluoromethoxyphenylhydrazene
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IGM	intra-cytoplasmic membrane
ISSO	insertion sequence 50
kb	kilobase pair
Ka	kanamycin
LHI	light-harvesting complex I
LPS	lipopolysaccharide
M	molar

mM	millimolar
$\mu$ Ci	microCurie
$\mu$ M	micromolar
$M_r$	molecular weight
mRNA	messenger ribonucleic acid
MCP	methyl-accepting chemotaxis protein
Nal	nalidixic acid
ng	nanogram
NPTII	neomycin phosphotransferase II
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PPO	2,5-diphenyloxazole
p.s.i.	pounds per square inch
PTS	phosphotransferase system
RNA	ribonucleic acid
RNase	ribonuclease
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
Sm	streptomycin
SSC	standard saline citrate
Tc	tetracycline
Tn	transposon
TPP <sup>+</sup>	tetraphenylphosphonium cation
Tris	Tris(hydroxymethyl)methylamine
U.V.	ultra violet
v/v	volume to volume
w/v	weight to volume

## CHAPTER 1

### INTRODUCTION



## 1 INTRODUCTION

### 1.1 Aims

The aim of this study was to examine the change from motile swarmer cell to non-motile reproductive cell as a landmark event in the differentiation cycle of the purple non-sulphur photosynthetic bacterium *Rhodomicrobium vannielii*.

In order to study changes in the expression of specific motility-associated proteins during the cell cycle, and ultimately how these changes are regulated, it was necessary to attempt to identify proteins of the flagellar apparatus and sensory transduction system. A brief description of the structure and organisation of the flagella and chemosensory mechanisms found in *Escherichia coli* and *Salmonella typhimurium* follows, as they have been intensively studied. These bacteria are peritrichously flagellated, i.e. flagella (about 6 in *E. coli*) originate at random points around the cell surface, but two other arrangements are found in other species. These are (i) a single flagellum, usually at one pole of the cell, as in *Caulobacter crescentus* and (ii) tightly arrayed polar tufts of flagella as in *Rhodospirillum rubrum*.

In this introduction I discuss firstly the structure of flagella and control of their regulation, and then introduce current research in prokaryotic differentiation, finally bringing these two areas together in a description of insights gained from the study of the *C. crescentus* cell cycle, and why I am interested in cellular differentiation in

*Rm. vannielii*.

## 1.2 Bacterial Flagella

### 1.2.1 Flagellar structures

Electron microscopy of flagellar organelles found on intact cells and in cell lysates has revealed that bacterial flagella are composed of three morphologically distinct substructures: the filament, the hook and the basal body (Silverman and Simon, 1977).

The filament is generally assembled from a single protein monomer, flagellin. Filaments are elongated by the addition of flagellin to the distal end, presumably by the passage of monomer down the length of the filament through a central cavity. The assembled filament is long (6-22  $\mu$ m), helical and semi-rigid, and bacteria swim by rotating it at its base (Berg and Anderson, 1973). Proton motive force, not ATP, is the energy source for this movement.

When the outer membrane, peptidoglycan layer and cytoplasmic membrane are dissociated, filament attached to hook-basal body complex is released (De Pamphilis and Adler, 1971a). Fig. 1.1 shows a model of the hook-basal body complex from *Escherichia coli* as deduced from electron micrographs.

The hook is also composed of a single polypeptide monomer, and anti-hook antiserum does not cross react with filament. Hooks are much more stable than filaments to denaturing agents such as low pH, high concentrations of urea and organic solvents.

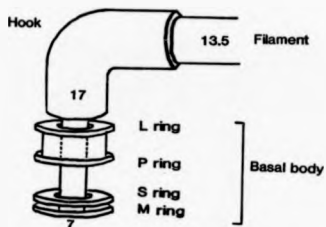


Fig. 1.1 Model of the basal body of a flagellum from *Escherichia coli*. Dimensions are in nanometres. From De Pamphilis and Adler (1971b).

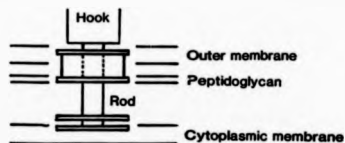


Fig. 1.2 Model of the attachment of the flagellar basal body of *Escherichia coli* to the cell envelope. From De Pamphilis and Adler (1971c).

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The basal body in *E. coli* and *S. typhimurium* consists of a stack of four rings with a central rod. De Pamphilis and Adler (1971c) deduced the relationship between the rings of the basal body and the components of the cell envelope, by preparing outer membrane with flagella attached, and examining them in the electron microscope. They used these results along with a knowledge of the structure and dimensions of the *E. coli* cell envelope and basal bodies from *E. coli* to construct the diagram shown in Fig. 1.2.

Other Gram negative bacteria such as *Caulobacter crescentus* and *Rhodospirillum rubrum* are different with respect to their basal body structure or relation between basal body and cell envelope. Fig. 1.3 shows the model for the attachment of *Rsp. rubrum* flagella to the cell envelope, as derived from electron microscope studies (Cohen-Bazire and London, 1967). They observed a "polar membrane" immediately adjacent to the cytoplasmic membrane at the pole of the cells, which surrounded the region of insertion of the flagellar tuft. In contrast, *C. crescentus* basal bodies have five rings (Johnson *et al.*, 1979), and Fig. 1.4 shows a diagrammatic representation of images seen in the electron microscope.

Flagella of the enteric bacteria have been used extensively as a model for studying the regulation of organelle morphogenesis. The study of mutants has been a very powerful tool in the elucidation of the order of assembly of component parts into a flagellum.

Motility mutants fall into three classes: *fla* mutants have no flagella, *mot* mutants have flagella but are unable to swim, and *che* mutants are chemotaxis deficient (Macnab and Aizawa, 1984). Using the gentle lysis procedure of De Pamphilis and Adler (1971a) to examine incomplete

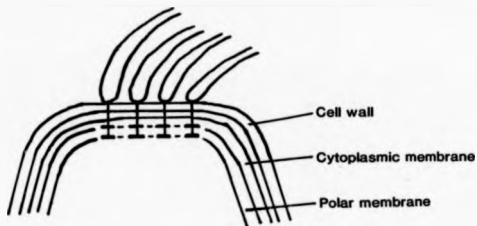


Fig. 1.3 Schematic representation of the relationship between the basal region of flagella and the cell envelope in *Rhodospirillum rubrum* deduced from electron micrographs by Cohen-Bazire and London (1967).

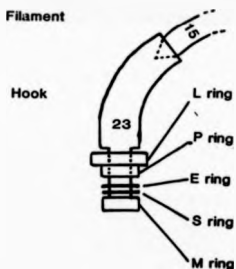


Fig. 1.4 Schematic model of the basal body complex of the *Caulobacter crescentus* flagellum. Dimensions are in nanometres. From Johnson *et al.* (1979).

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flagellar structures from specific *S. typhimurium* (Suzuki et al., 1978) and *E. coli* (Suzuki and Komeda, 1981) *fla* mutants in the electron microscope, it was deduced that assembly proceeded step by step from simpler structures to more complex ones. The sequence appeared to be rod-M ring-S ring, rod-M ring-S ring-P ring, complete basal body, basal body-hook and finally basal body-hook-filament.

#### 1.2.2 Regulation of expression of flagellar genes in *Escherichia coli* and *Salmonella typhimurium*

Synthesis of the flagellar organelle in *E. coli* is prevented by the presence of glucose, i.e. it is sensitive to catabolite repression.

Catabolite repression was discovered as a phenomenon whereby the presence of a rapidly metabolisable substrate (e.g. glucose for *E. coli*) repressed the synthesis of inducible enzymes for the uptake and catabolism of more slowly metabolisable carbon and energy sources, and the following is an outline of how it works. The amount of ATP available to a cell is inversely proportional to the intracellular concentration of 3'5'-cyclic adenosine monophosphate (cAMP), which is synthesised by the enzyme adenylyl cyclase, encoded by the gene *cya*. Binding of cAMP to cAMP receptor protein (CRP), encoded by the gene *crp*, causes the protein to undergo an allosteric conformational change which allows it to bind to the chromosome near the genes encoding catabolite repressible enzymes or structural proteins, and through such binding to stimulate transcription of these genes.

Mutants lacking adenylyl cyclase or CRP are also unable to synthesise flagella. If exogenous cAMP is added to either wild type cells in the presence of glucose or the *cya*<sup>-</sup> cells, the effect can be reversed and

flagella are synthesised.

Mutants could be isolated called *cfs* (constitutive flagella synthesis) which allowed flagellar synthesis to occur in *cys*<sup>-</sup> strains, *crp*<sup>-</sup> strains and strains grown in the presence of catabolite repressing substrates (Silverman and Simon, 1974). Deletion studies showed that the mutation was in or very near the *flaI* gene. Similar regulation was observed in *S. typhimurium* (Komeda *et al.*, 1975). This was the first evidence for a gene exerting a positive control effect on the expression of other flagellar genes, and led to the discovery of the cascade regulation of flagellar genes through the study of *lac* gene fusions.

The structural and regulatory components of flagella have no assayable activity other than integration into the whole organelle. For this reason Komeda and Iino (1979) constructed a *hag::Mud(Ap<sup>r</sup>lac)* mutant which had promoterless *lac* genes fused to the promoter of the *E. coli* flagellin gene (*hag*). This was done using the bacteriophage *Mud(Ap<sup>r</sup>lac)* developed by Casadaban (1976). This allowed measurement of flagellin gene expression by assaying  $\beta$ -galactosidase activity.

When the *hag-lac* fusion was manipulated such that it was in the *fla*<sup>-</sup> background of each of 27 different *E. coli* *fla* mutants it was found that with the great majority of the mutants (23), flagellin was not transcribed. Thus 23 of the *fla* genes tested were required for flagellin transcription; only 4 of the *fla* mutations allowed transcription of the *hag-lac* fusion.

More recently, the work of Komeda (1982 and 1986) has made a large contribution to the understanding of transcriptional control of

flagellar genes in *E. coli*. This has allowed 34 *fla*, *mot* and *che* genes in 13 operons to be grouped into 6 groups or regulons. The genes for completion of a flagellum comprise around 3% of the *E. coli* genome, and the regulation of transcription of these genes and the interaction of their protein products are very complex. However, the data from extensive *lac* fusion studies have elucidated that the 6 groups or regulons are part of a regulatory cascade such that, for example, the transcription of groups 4, 5 and 6 is repressed until all the proteins of group 3 are successfully synthesised. The evidence is that the correct folding and integration of proteins into the organelle at each stage of its assembly is important for transcription of the next genes in the cascade.

The gene for flagellin, *hag*, is the last gene in the cascade, and all along there is agreement between the order of transcription of the genes of the regulatory cascade with the order of assembly derived from electron microscopy of incomplete flagellar structures from characterised *fla* mutants.

#### 1.2.2.1 Flagellar phase variation in *Salmonella typhimurium*

When the flagellar antigen of *Salmonella typhimurium* cells derived from a single colony is characterised serologically, then the clone is allowed to continue growth, a second, different flagellar antigen serotype can be detected. When a cell is selected that has the second serotype, it can give rise on further growth to the first serotype, i.e. individual *S. typhimurium* cells have the capacity to alternate between two flagellar antigens or phases. The transition occurs with a low frequency per bacterial division, ranging from  $10^{-3}$  to  $10^{-5}$ , and is thought to aid pathogenic *S. typhimurium* in the evasion of host immune



responses.

Phase variation has been characterised genetically and at the molecular level. It is the result of mutually exclusive expression of two flagellin genes, *H1* and *H2*. The *H2* gene and the gene for a repressor of *H1* expression exist as an operon, and the promoter for this operon is located on an invertible segment of DNA around 1000 base pairs long. Also encoded within this invertible segment is the *hin* gene product, an enzyme which catalyses inversion of the invertible segment by site-specific recombination. When the invertible segment is in one orientation, the promoter drives transcription of the *H2* flagellin gene and the gene for the repressor of *H1* synthesis. In the other orientation, the promoter is uncoupled from the *H2* operon, and *H1* flagellin is synthesised from its own promoter. Thus, the regulatory mechanism for flagellar gene switching is the reversal of polarity of a promoter element by DNA rearrangement.

It is thought that the switch in phenotype does not necessarily require an inducing event, rather that switching constantly occurs in the population at a low frequency. However it is possible that presently undetermined host factors can regulate the frequency of the site-specific recombination event (Silverman and Simon, 1983).

### 1.3 Bacterial Chemotaxis

Motile bacteria are attracted by certain chemicals and repelled by others. They respond to spatial gradients by monitoring concentration as a function of time, and the phenomenon is called chemotaxis.

*Escherichia coli* and *Salmonella typhimurium* motility is characterised by periods of smooth swimming, during which the flagella rotate counter-clockwise, interrupted by chaotic tumbling, when the direction of rotation of the flagella is switched to clockwise. This produces the "random walk" seen in the light microscope.

When an attractant is added, the cells repress tumbling, in order not to swim away from it, then, after a lag period, the frequency of tumbling returns to the pre-stimulus level, i.e. the cells adapt to the repeated stimulus. Addition of a repellent, and removal of an attractant, cause a short period of increased tumbling (Springer *et al.*, 1979).

Adler (1969) found that metabolism of attractants in *E. coli* is not required for chemotaxis, and deduced that the attractants themselves are detected by chemoreceptors.

#### 1.3.1 Methyl-accepting chemotaxis proteins

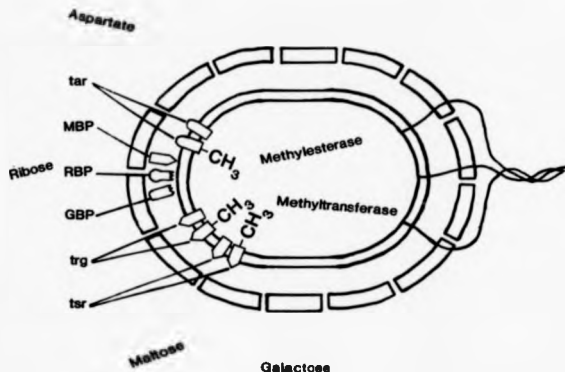
Methyl-accepting chemotaxis proteins (MCPs) were found in *E. coli* and *S. typhimurium* through the discoveries that the extent of covalent methylation of several cytoplasmic membrane proteins was altered by some chemicals that elicited a chemotactic response, and that this methylation reaction failed to occur in some non-chemotactic mutants.

MCPs are now known to be transmembrane proteins located in the cytoplasmic membrane, with a molecular weight of around 60k in the coliform bacteria. They are capable of being methylated at the  $\gamma$ -carboxyl group of one or more glutamate residues.

The basal level of methylation of an MCP is rapidly increased when an attractant is added to cells in the laboratory, and remains high until the attractant is removed. Addition of a repellent causes a decrease in methylation from the basal level, and removal of a repellent causes remethylation to the basal level. It is thought that when either the chemoattractant itself, or the chemoattractant bound to its periplasmic binding protein, binds to the periplasmic side of the MCP, a conformational change is induced in the MCP such that carboxyl groups on its cytoplasmic side, previously shielded, become exposed to a cytoplasmic methyltransferase (Koshland, 1981).

The conformational change in the MCP results in both sensory excitation and sensory adaptation. Adaptation is the process which allows the cell to become desensitised to a repeated external signal in order to de-emphasise that signal in relation to others, and is necessary for a biological sensory system to fulfil the needs of an organism satisfactorily. That the function of methylation of MCPs is in the adaptation process has been shown by study of methylation deficient mutants. Stock *et al.* (1985) have found that there is an alternative adaptation system operating in *S. typhimurium* which may be used for adapting to small changes in concentration of attractants or repellents).

At present unknown (Kuo and Koshland, 1987), there is some parameter, referred to as the rotation regulator, which increases when an attractant is added, and signals to the flagella to rotate only counter-clockwise. At the same time, addition of an attractant also causes a relatively slow increase in the methylation of the appropriate MCP, allowing adaptation to occur.



**Fig. 1.5** Diagram of the methyl-accepting chemotaxis protein (MCP) bacterial sensory system. MBP is maltose binding protein, RBP is ribose binding protein and GBP is galactose binding protein. The MCP for aspartate and maltose is a product of the *tar* gene in *E. coli*, the MCP for ribose and galactose is a product of the *trg* gene in *E. coli*, and the MCP for serine is a product of the *tsr* gene. Modified from Koshland (1981).

Fig. 1.5 shows a schematic summary of the bacterial sensing system described above. Examples of compounds that are chemoattractants sensed by this system are ribose, galactose, maltose, serine and aspartate.

### 1.3.2 Phosphotransferase system enzymes as chemoreceptors

The phosphotransferase system (PTS) supplies chemoreceptors for sugars such as glucose, mannose and fructose which do not have soluble periplasmic binding proteins.

The PTS, so called because phosphorylation is coupled to the transport of certain sugars, consists of a number of enzymes. Enzyme I and HPr (a phosphate carrier protein) are cytoplasmic proteins involved in the transport of all PTS carbohydrates, and enzymes II are sugar specific inner membrane bound enzymes (Postma, 1986). As shown in Fig. 1.6, enzyme I is covalently phosphorylated at a histidine residue at the expense of phosphoenolpyruvate, and catalyses the phosphorylation of the HPr protein, also at a histidine residue. The appropriate enzyme II then catalyses the phosphorylation of the sugar by the phosphorylated HPr protein. (With certain sugars another soluble protein is involved.)

There are two distinct enzymes II for D-glucose in *E. coli* with different specificities. One phosphorylates D-glucose and not D-mannose, and is referred to as glucose enzyme II, whereas the other phosphorylates glucose and mannose, and is referred to as mannose enzyme II. Adler and Epstein (1974) used a parental strain lacking the periplasmic galactose binding protein, as this protein binds glucose and allows chemotaxis to glucose, in order to isolate mutants lacking glucose enzyme II, mannose enzyme II, or both. They found that mutational loss of an enzyme II results in loss of taxis towards the

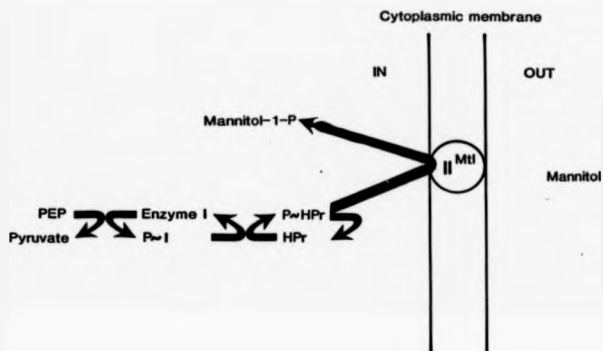


Fig. 1.6 Diagram of part of the phosphoenolpyruvate: carbohydrate phosphotransferase system. Modified from Postma (1986).

sugar it transports. They concluded that there is a very close link between chemotaxis and the transport process, although transport is not necessary for an enzyme II to act as a chemoreceptor.

Langeler et al. (1981) confirmed the work of Adler and Epstein by failing to find a mutant which had enzyme II catalysed transport without chemotactic activity despite an intensive search. They proposed that the decisive stimulus in enzyme II-mediated chemotaxis is the reversible alternation of this enzyme between the phosphorylated and dephosphorylated states, i.e. reversible covalent modification, also found with the MCPs.

Vogler and Langeler (1987) showed, by careful examination of mutations mapping in the *crp* locus of *E. coli*, that the mutation apparently prevented expression of a hitherto unidentified molecule involved in enzyme II-mediated signal transduction.

### 1.3.3 Aerotaxis, phototaxis and the role of membrane potential in signal transduction

Molecular oxygen elicits aerotactic responses in most motile bacteria. In general, strict aerobes migrate towards oxygen, microaerophiles accumulate where there is an appropriately low concentration of oxygen, and strict anaerobes are repelled by oxygen.

Motile purple bacteria reverse their swimming direction when they leave an area illuminated with photosynthetically effective light, and so accumulate in a spot of light projected on a cell suspension in the laboratory. This phototaxis employs bacteriochlorophyll and carotenoids as receptors, and requires the photosynthetic reaction centre (Taylor,

1983). Enteric bacteria are repelled by potentially harmful blue light (Taylor *et al.*, 1979).

Taylor *et al.* (1979) studied taxis of *E. coli* and *S. typhimurium* towards respiratory terminal electron acceptors, among them  $O_2$ . They concluded that changes in membrane potential form part of a pathway for signal detection and transduction by the cell, resulting in tumbling when the p.m.f. drops and smooth swimming when it increases. Another conclusion they reached was that the receptor for taxis towards oxygen is the terminal component of the electron transport pathway, which is a cytochrome. The receptor for taxis towards anaerobic respiratory terminal electron acceptors was also the appropriate terminal component of the electron transport pathway.

Harayama and Iino (1977) found that phototaxis in *Rhodospirillum rubrum* is mediated by the sudden change in electron flow in the photosynthetic electron transfer system when a dark area is entered, and showed that membrane potential plays an important role in the response. They did this using an ionophore at concentrations which partially collapsed the membrane potential, without abolishing motility. They observed that the phototactic activity of the cells was inhibited under these conditions.

These findings led Taylor (1983) to propose that p.m.f. mediates only those behavioural responses that are directly involved in maintaining energy balance, e.g. aerotaxis in aerobic bacteria and phototaxis in photosynthetic bacteria.



#### 1.4 Morphogenesis and Differentiation in Bacteria

The majority of prokaryotes do not exhibit extensive morphogenesis and differentiation which can be detected in the light or electron microscope, or by visual observation of cultures on solid media. Those which do are the subject of intensive research, firstly because the biology of these organisms is invariably interesting and attractive, and secondly because elegant techniques available for the genetic manipulation of bacteria can yield much information on the biochemistry and physiology of the differentiation process.

Differentiation in the Gram negative freshwater bacterium *Caulobacter crescentus* proceeds as a series of cell cycle dependent morphological changes, including a swarmer cell to stalked cell transition, and is described in detail in section 1.5.

Among other bacteria that have been used in the study of microbial differentiation are the endospore-forming bacteria of the genus *Bacillus* (Losick *et al.*, 1986), the heterocyst-forming cyanobacteria (Golden *et al.*, 1985), bacteria of the genus *Myxococcus* which show social interaction and multicellular development (Kaiser, 1986), and bacteria of the *Streptomyces* genus, which grow as mycelia (Hopwood *et al.*, 1986).

There follows a brief description of examples of these, designed to highlight techniques being used to answer questions about microbial differentiation. For detailed descriptions of research based on these differentiation systems, see the reviews cited.

#### 1.4.1 Bacillus subtilis

*Bacillus subtilis* is a Gram positive heterotrophic bacterium, and can be triggered to form an endospore which is resistant to heat, radiation, organic solvents and lysozyme, in response to carbon, nitrogen or phosphorous starvation. Endospore formation takes 8-10 hours at 37°C.

It is thought that reduction in the intracellular concentration of GDP and GTP, or a closely related molecule, is the initiating signal for differentiation, although it is not known how the cell senses the guanine nucleotide concentration (Losick *et al.*, 1986).

Once initiated, sporulation proceeds through six or seven defined morphological stages (Fig. 1.7), culminating in the formation and release of the mature endospore. The vegetative cell shown at stage 0 contains two chromosomes, which separate at stage II, and asymmetric septation occurs. Engulfment (stages II-III) gives rise to a two cell organism in which one cell is contained within another. The two cells interact, and have different fates (Mandelstam and Errington, 1987).

In parallel with examination of the morphological changes occurring during sporulation, the biochemistry of how resistance is conferred by sporulation has been studied. Heat resistance is due to the anhydrous condition of the core of the spore, which is filled with calcium dipeicolinate, a compound unique to endospores. Resistance to ultraviolet light has been attributed to the association of a family of acid soluble proteins with spore DNA, and resistance to lysozyme and organic solvents is due to the barrier created by the outermost polypeptides of the spore coat.

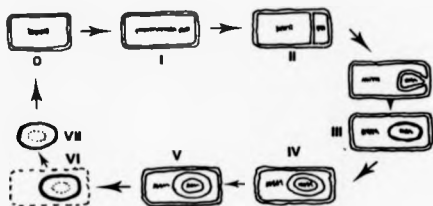


Fig. 1.7 Stages of sporulation in *Bacillus subtilis* (from Losick et al., 1986).

Genetic approaches have aided research into the programmed developmental gene expression required for endospore formation. In fact, understanding of the biochemical changes which determine morphological events during endospore formation currently lags behind understanding of the genetics of the process. A large number of sporulation mutants have been isolated which are arrested at a specific stage in sporulation. These *spo* mutations have been mapped, and genes of the same developmental stage are found at widely scattered sites on the chromosome.

The transposon Tn917, which was discovered in bacteria from another Gram positive genus, *Streptococcus*, carrying a gene for erythromycin resistance, can be used as a random chromosomal mutagen in *B. subtilis* (Youngman *et al.*, 1985). This has facilitated cloning of developmentally regulated genes from *Bacillus* spp. in *E. coli*.

In a similar way to that described for *E. coli* (section 1.2.2), expression of genes not possessing a readily assayable phenotype can be assessed using gene fusions in *B. subtilis*. Many genes mutated by Tn917 insertions can be fused to a promoterless *E. coli*  $\beta$ -galactosidase gene (*lacZ*) using a fusion generating transposon constructed from Tn917 called Tn917 *lac*. In this case, the *lacZ* gene was furnished with a ribosome binding site appropriate for efficient translation in *B. subtilis*, rendering  $\beta$ -galactosidase activity indicative of transcription from a *Bacillus* promoter, when the transposon integrated in the right orientation.

*lacZ* fusions and Northern blotting were used to assay expression of cloned genes in experiments to determine the order of expression of

sporulation genes and operons. It became apparent that there was a dependent sequence of gene expression, or regulatory cascade, but that the sequence was branched so that some operons were required to be expressed in parallel to allow expression of an operon needed later in sporulation (Mandelstam and Errington, 1987).

Biochemical studies involving *in vitro* transcription and genetics have contributed to the discovery that the DNA dependent RNA polymerase of *B. subtilis* can exist in six or more different holoenzyme forms.

Holoenzyme consists of the core polymerase plus a sigma subunit. The sigma subunit confers on core polymerase the capacity to recognise and initiate transcription from promoter sequences on DNA. Each of the multiple holoenzyme forms of *B. subtilis* RNA polymerase contains a different sigma factor species that confers specificity for a characteristic class of promoters, and there is evidence that other protein factors which interact with specific holoenzymes are required for correct regulation of gene expression (Losick *et al.*, 1986).

It is now becoming possible to look for DNA sequences which are characteristic of developmentally regulated genes, as many sporulation genes have been cloned. Regions of DNA which are necessary for transcription, and others which enhance transcription have been defined.

Scientists in the *Bacillus* sporulation field now feel that the regulatory networks coordinating the program of sporulation gene expression will be understood in the near future (Losick *et al.*, 1986; Mandelstam and Errington, 1987).

#### 1.4.2 *Anabaena* spp.

Several genera of cyanobacteria differentiate specialised cell types.

Heterocysts are found in species of *Anabaena*, *Calothrix* and *Nostoc*, and akinetes occur in members of the families *Nostocaceae*, *Rivulariaceae* and *Stigonemataceae* (Dow *et al.*, 1983).

*Anabaena* spp. are filamentous cyanobacteria, and many form heterocysts and akinetes. Akinetes have greater resistance to cold and desiccation, and it is thought that they function as dormancy structures for survival in hostile environments. Germinating akinetes rupture and release vegetative cells which have already divided, and in the case of *Anabaena* sp. CA, a filament of several vegetative cells with an intercalary heterocyst is released. There is evidence for light being the factor which induces germination of akinetes (Dow *et al.*, 1983).

Much recent research has centred on heterocyst differentiation.

*Anabaena* grows photoautotrophically using green plant-like  $O_2$ -evolving photosynthesis and is capable of fixing dinitrogen. Heterocysts function as anaerobic compartments for nitrogen fixation under external aerobic conditions. They do this by synthesising a multilayered envelope exterior to the cell wall to reduce the entry of gases, and reorganising the photosynthetic apparatus to shut down the  $O_2$ -evolving activity of photosystem II while retaining the ATP-synthesising activity of photosystem I (Haselkorn, 1978).

When *Anabaena* spp. such as *Anabaena cylindrica* or *Anabaena* 7120 are grown in, or transferred to, medium lacking fixed nitrogen in the form of ammonia or nitrate, heterocysts differentiate at regular intervals along the filament, approximately every tenth cell. Then, the extremely

O<sub>2</sub> sensitive enzymatic machinery for nitrogen fixation is contained in the heterocyst. Glutamine, the product of nitrogen fixation is exported from heterocysts to vegetative cells, and reduced carbon is exported from vegetative cells to heterocysts (Haselkorn, 1978). Interest in heterocyst differentiation has centred on the biochemistry of development of these specialised cells and the control of heterocyst spacing along the filament.

Recently, analysis of vegetative cell DNA and heterocyst DNA from *Anabaena* 7120 by Southern blotting, using specific fragments of DNA carrying nitrogen fixation genes as probes, has revealed that the terminal differentiation process of heterocyst formation is accompanied by DNA rearrangements in the genome of the differentiating heterocyst. In one of the rearrangements an 11 kb fragment of DNA is excised during a site-specific recombination event, bringing nitrogen fixation genes which are 11 kb apart in the vegetative cell genome and not expressed, close together in the heterocyst genome where they are expressed (Golden *et al.*, 1985). Heterocyst differentiation takes 30 hours, and this occurs late on in the process.

Other examples of DNA rearrangements occurring under the control of external or developmental cues have been documented, in prokaryotes and eukaryotes (Golden *et al.*, 1985).

#### 1.4.3 *Myxococcus xanthus*

Myxobacteria of the genus *Myxococcus* have been studied with respect to cellular social interactions and the multicellular development of fruiting bodies containing spores.

They are Gram negative soil bacteria which move with gliding motility on a solid surface. They divide by binary fission, and feed on other bacteria by secreting several proteases, lysozyme and other bacteriolytic enzymes.

Amino acid starvation triggers fruiting body development in *M. xanthus*. The program of development is divided into three stages: initiation, intercellular signalling and aggregation. Many thousands of cells glide to aggregation centres where they mound on top of each other. Following aggregation, some cells in each mound lyse, while others differentiate into dormant, non-motile thick-walled myxospores resistant to hostile environments (Kaiser, 1986).

Thus, *M. xanthus* provides the opportunity to examine morphogenesis of a multicellular structure, and intercellular communication in a prokaryote, as well as the development of a myxospore from a vegetative cell.

Selection for failure to sporulate during fruiting body development led to the isolation of a set of mutants that were defective in intercellular signal production. They grow normally, but are conditionally defective in development, in that developing wild type cells can provide the missing signal, allowing the mutants to complete their development. The mutants fall into four extracellular complementation classes.

It is possible to use a *Tn5* based promoter probe carrying a promoterless *lacZ* gene in *M. xanthus*. A set of strongly developmentally regulated *Tn5 lac* insertions has been isolated, and they are expressed over a wide



range of times during the development of a fruiting body, which takes one day.

By introducing different developmentally regulated Tn5 *lac* insertions into the mutants defective in signal production, it has been found that expression of certain developmentally regulated genes is again dependent on the expression of other developmentally regulated genes (Kaiser, 1986).

#### 1.4.4 Streptomyces

*Streptomyces* are Gram positive filamentous soil bacteria which grow on a wide variety of carbon and energy sources. The characteristic odour of damp soil is attributable to a volatile substance which they produce. Interest in *Streptomyces* stems from their complex cycle of morphological development and their ability to synthesise a huge variety of metabolites with antibiotic or other interesting biological effects (Hopwood, 1986).

The *Streptomyces* developmental cycle is illustrated in Fig. 1.8. Germination of a spore occurs by hyphal outgrowth to produce a substrate mycelium. A looser aerial mycelium then arises over the surface of the colony, the hyphae of which have a fibrous sheath. During vegetative growth few cross walls are formed, but before sporulation the aerial hyphae coil and many septa form within them. Spores arise exclusively on the aerial mycelium through fragmentation of the hyphal tip within its sheath, resulting in chains of spores enclosed by a common sheath. At maturity the spores are separated from each other and released, each enclosed in a section of sheath layer (Stanier *et al.*, 1977).

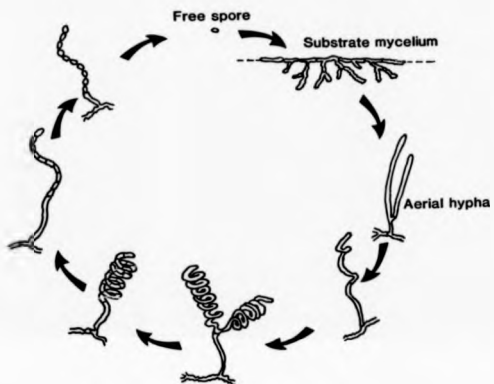


Fig. 1.8 Development of *Streptomyces coelicolor*. Modified from Hopwood et al. (1986).

It appears from recent research that multiple species of RNA polymerase sigma factor play an important role in gene selection for transcription in *Streptomyces*. Also, a feature of the *Streptomyces* genes analysed so far is the common occurrence of pairs of tandem promoters (Hopwood, 1986). This has also been observed with one of the sporulation genes from *Bacillus subtilis*. In the *Bacillus* case it is known that the transcription initiation region is composed of two overlapping promoters that are separately utilised by forms of RNA polymerase that contain different species of sigma factor (Youngman *et al.*, 1985).

One other similarity is that sporulation is also associated with antibiotic production in *Bacillus* spp., and common mechanisms for the control of differentiation may be present in these Gram positive bacteria.

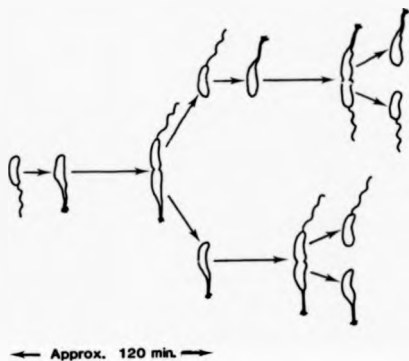
### 1.5 Differentiation in the *Caulobacter crescentus* cell cycle

Unlike *Bacillus*, *Myxococcus* or *Anabaena*, where differentiation is initiated according to environmental cues sensed by the bacterium, differentiation in *Caulobacter* is coordinated with the cell cycle when the bacteria are in the exponential phase of growth. Stove and Stanier first documented this in 1962; previously it had been thought that stalk formation was a facultative event in the life cycle, occurring when it was advantageous for the bacterium to have a stalk and holdfast attaching it to a solid surface. By microscopic observation of the development of synchronised populations of swarmer cells and stalked cells respectively, Stove and Stanier found that stalk formation is an obligatory stage in the development of every swarmer cell, attached or

unattached, and always precedes the first division. Fig. 1.9 illustrates the *Caulobacter crescentus* cell cycle. The reproductive cell is the non-motile cell bearing a polar prostheca or stalk. This cell elongates, and before division, a flagellum is formed at the pole opposite the stalk, then binary fission produces dissimilar daughter cells: a non-motile stalked cell and a flagellated non-prosthecae swarmer. The prosthecae cell can divide again in the same manner, but the swarmer cell is incapable of division until it has shed its flagellum and synthesised a prostheca at the same site.

This defined series of morphogenetic events has been studied with a view to elucidating firstly how the cell controls the sequence of specific changes, i.e. the temporal regulation of differentiation, and secondly, how the cell controls the biogenesis of, for example, the prostheca or flagellum at a specific site on the cell envelope, i.e. spatial regulation of differentiation. (In addition to the prostheca and flagellum, there are pili and phage receptor sites that are present only during a specific period of the cell cycle (Shapiro, 1976).)

In order to study differentiation in *Caulobacter crescentus* it is necessary to obtain synchronised populations of cells. This has been achieved by several methods, one of which involves layering a heterogeneous population of cells onto a Ludox (a colloidal silica obtainable from Dupont) density gradient which is then briefly centrifuged. Swarmer cells have a higher buoyant density than the stalked cell population and so can be separated from a heterogeneous culture. After centrifugation the isolated swarmer are washed in culture medium, and are subsequently found to differentiate normally and synchronously (Evinger and Agabian, 1977).



**Fig. 1.2** The *Caulobacter crescentus* cell cycle, from continuous microscopic observations of clonal growth performed by Stove and Stanier (1962).

Much of the following concerns research into the synthesis and assembly of the flagellum at a specific period during the cell cycle and at a specific site on the cell.

1.5.1 Characterisation of the *Caulobacter crescentus* flagellum and the periodic synthesis of flagellins and hook protein

*C. crescentus* flagella are unusual in that they are composed of at least two different flagellin subunits, flagellin A, molecular weight 25k, and flagellin B, molecular weight 27.5k. There is another *C. crescentus* flagellin precipitated by anti-flagellin antibody, molecular weight 29k, which may function transiently in flagellar filament assembly or may be a very minor component (Gill and Agabian, 1983). Most bacterial flagella so far examined consist of only one protein subunit.

The ratio of flagellin A to flagellin B in shed flagella is 4:1. Both proteins are present in all flagella, but flagellin B predominates at the hook proximal region, while the tip of the filament is composed purely of flagellin A. Between the extremities of the filament there is a region composed of both flagellins. The two proteins are immunologically cross-reactive, but are encoded by separate genes. Each flagellin is capable of reaggregation *in vitro* to form filaments identical to those formed *in vivo*, but the flagellins do not seem to be interchangeable *in vivo* as both are required for formation of a full length flagellum (Weisborn *et al.*, 1982). However, mutants carrying a deletion in the gene encoding the 29k flagellin are able to assemble a flagellum and are motile. The 29k flagellin is thus not essential for motility, but these mutant strains form colonies which are smaller than those formed by the wild type strain (Loewy *et al.*, 1987). The 29k flagellin is the first flagellin to be synthesised (Minnich and Newton,

1987).

Lagenaur and Agabian (1978) have shown by radioimmune precipitation of labelled flagellins from synchronously developing cells that flagellin synthesis varies markedly during the cell cycle, and that the relative concentrations of the two flagellins vary with respect to each other. The major period of flagellin synthesis occurs in the predivisional cell and the swarmer cell, i.e. coincident with the appearance of the flagellum and consequent motility. Flagellin A is synthesised in the predivisional cell and the swarmer cell, but synthesis of flagellin B is confined to the predivisional cell. How this switching on and off of flagellin synthesis is controlled has been intensely studied.

In *C. crescentus* the hook subunit is a protein of approximately 70k D<sub>r</sub> (Lagenaur *et al.*, 1978; Sheffery and Newton, 1979, Johnson *et al.*, 1979). Lagenaur and Agabian (1978) found that the major period of hook protein synthesis corresponded to the onset of synthesis and assembly of the flagellin subunits.

Hook basal body complexes were isolated (Johnson *et al.*, 1979) using a modification of the gentle lysis procedure developed by De Pamphilis and Adler (1971a). Fig. 1.4 shows a diagram of these complexes derived from electron micrographs. The fact that the *C. crescentus* basal body has 5 rings (an extra inner ring, called the E ring by Mahnenberger and Shapiro (1987), in comparison with *E. coli*), along with their finding that *C. crescentus* periodically sheds filament, hook and rod into the medium, led the authors to speculate that the extra ring may be involved in the release mechanism. Whether this is so will probably not be determined until the molecular biology of basal body formation is

elucidated.

Thus, the spatially regulated biogenesis of surface structures is superimposed on the usual prokaryotic cell cycle of initiation of DNA synthesis, DNA synthesis, septation and cell division in *C. crescentus*. A mutant which failed to make flagella or pili, but could still grow and divide, indicated that cell division occurs independently of the assembly of surface structures. This mutant also showed that the expression of polarity was independent of the assembly of surface structures, as stalk synthesis occurred at the proper site and at the correct time in the cell cycle (Shapiro, 1976).

By measuring the incorporation of tritium labelled guanosine into DNA in a synchronous culture, and assaying for flagellin synthesis using radioimmune precipitation from a synchronous culture, Osley *et al.* (1977) established when, in relation to the cell cycle, flagellin synthesis occurs. It was revealed that flagellin synthesis increased abruptly shortly before the end of DNA synthesis. In addition they presented evidence that it is necessary for *C. crescentus* to complete about half of its chromosome replication in order to be able to express its flagellar genes.

Osley *et al.* (1977) also showed that if rifampicin, which inhibits initiation of transcription in *C. crescentus*, is added to a synchronous culture at any time before the cells are half way through DNA synthesis, the synthesis of flagellin is completely blocked, i.e. transcription is required for flagellin synthesis in the predivisive cell.

Sheffery and Newton (1981) confirmed that the expression of the



flagellins is independent of cell division, using the radioimmune precipitation assay in the presence of concentrations of penicillin which prevent cell division, but allow normal growth and DNA synthesis.

It was also found by Osley *et al.* (1977) that the half-lives of the mRNAs for flagellin A and flagellin B are different. They calculated mRNA half-lives by measuring the residual rate of flagellin synthesis at different times after the addition of rifampicin. A half-life of 6.5 minutes was found for flagellin A mRNA, and 2.5 minutes for flagellin B mRNA; the half-life of the bulk mRNA in exponentially growing cells of *C. crescentus* being 2.5 minutes. They proposed that the longer functional half-life of flagellin A mRNA explains why flagellin A is synthesised in swarmer cells after division in the absence of transcription. This suggests that the flagellin made in swarmer cells is translated from mRNA transcribed before cell division.

By analysing membrane phospholipid synthesis in *C. crescentus*, Contreras *et al.* (1980) examined whether a specific relationship exists between membrane synthesis and the expression of cell cycle events. They found that inhibition of phospholipid synthesis resulted in the cessation of DNA replication and that upon termination of phospholipid synthesis swarmer cells shed their flagella, initiated stalk formation, but stalk elongation failed to occur. When anti-flagellin and anti-hook antibody was used to prepare immunoprecipitates from a heterogeneous culture at various times after inhibition of phospholipid synthesis, it was found that the synthesis of flagellin A, flagellin B and hook protein was inhibited after one hour, whereas synthesis of other proteins continued for at least four hours. Cell division was blocked when phospholipid synthesis was inhibited. The authors concluded that the specific

inhibition of synthesis of flagellar proteins is probably due to the inhibition of DNA synthesis soon after termination of phospholipid synthesis; thus their data are in agreement with those of Osley *et al.* (1977). As a consequence of these and other observations there has been a great deal of interest in whether interaction between the growing membrane and the replicating chromosome can help account for the spatial distribution of cell components (Shapiro *et al.*, 1982). Osley *et al.* (1977) proposed that the position of a gene on the chromosome could be important both in the timing of its expression, and the location of its product in the cell. They suggested that expression of a gene could be switched on when it is replicated, and that the timing of the synthesis of the differentially expressed protein could be such that it is physically located near a defined site on the growing membrane.

#### 1.5.2 Control of expression of genes required for motility and chemotaxis

DNA dependent RNA polymerase from *C. crescentus* swarmer, predivisional and stalked cells was prepared by Bendis and Shapiro (1973). No differences in subunit composition or molecular weight were detected, and only minor differences in template preference could be detected. They concluded that, within the limitations of the methods used to analyse the enzyme, major changes in the RNA polymerase cannot account for changes in gene expression associated with differentiation in *C. crescentus*, although it cannot be ruled out that minor species of sigma factor are present, but are invisible on a Coomassie blue stained polyacrylamide gel. As a result of their findings, work aimed at the elucidation of control of gene expression has centred on the use of specific cloned genes, promoter probes and DNA sequencing.

#### 1.5.2.1 Methyl-accepting chemotaxis proteins

Shaw *et al.* (1983) showed that the methyl-accepting chemotaxis protein (MCP) chemosensory apparatus (section 1.3.1) operates in *C. crescentus*. They characterised *C. crescentus* MCPs as a set of several membrane proteins with molecular weights between 74k and 92k.

MCP methylation is easily assayable *in vivo* and *in vitro* by measuring transfer of tritiated methyl groups from [methyl-<sup>3</sup>H] methionine to MCPs (section 2.27 and Shaw *et al.*, 1983). Conversely, methyltransferase activity can be assayed *in vitro* by incubating membranes containing [methyl-<sup>3</sup>H] labelled MCPs with test soluble extracts, and measuring the decrease in tritium in the MCPs (Gomes and Shapiro, 1984). Consequently these assays give access to the study of the temporal and spatial regulation of a set of motility associated proteins during the cell cycle.

Labelling synchronised *C. crescentus* cells with [methyl-<sup>3</sup>H] methionine at various times during the cell cycle showed that MCP methylation occurs only in the swarmer and the predivisional cell. *In vitro* assays confirmed that these cells contained MCPs and methyltransferase activities, while the stalked cell possessed neither activity (Shaw *et al.*, 1983).

When it was determined that the activities required for MCP methylation were regulated during the cell cycle, Gomes and Shapiro (1984) addressed the question of whether the synthesis of the MCPs, methyltransferase and methyltransferase is switched on and off during differentiation; or are the proteins present constitutively, and switched between active and inactive forms during the cell cycle?

They used antisera raised against (i) *S. typhimurium* methylesterase and (ii) the *S. typhimurium* MCP encoded by the *tar* gene to answer this question. Samples taken periodically during the synchronous development of a *C. crescentus* population were labelled with  $^{14}\text{C}$  amino acids, and cell free extracts of the samples were immunoprecipitated. The *S. typhimurium* anti-MCP antibody precipitated at least three *C. crescentus* MCPs, and showed that *C. crescentus* MCPs were synthesised only in the predivisive cell. The *S. typhimurium* anti-methylesterase antibody precipitated a 36k protein, among a few others, from *C. crescentus* cell free extracts. Evidence was presented that this was the *C. crescentus* methylesterase, and it was also synthesised only in predivisive cells. Thus, the methylesterase and the MCPs are synthesised concomitantly with the synthesis of the components of the flagellum in the predivisive cell. A summary of the times of synthesis of some motility-associated proteins during the cell cycle is shown in Fig. 1.10.

The differences between the period and location of (a) the activity of the proteins and (b) the synthesis of the proteins has implications in the control of positioning of cell components and will be discussed in section 1.5.3.

#### 1.5.2.2 Cloning of developmentally regulated genes from *Caulobacter crescentus*

Johnson and Ely (1979) isolated 69 spontaneous nonmotile mutants, the majority of which were *fla* mutants, although 8 synthesised a short stub of a flagellum and 4 were *mot* mutants. There is available a generalised transducing phage for *C. crescentus*, called  $\phi\text{Cr30}$ , which facilitated the mapping of these mutations. The *fla* mutations fell into 27 linkage groups and the *mot* mutations into 3. *C. crescentus fla* genes were found

	100	10	100			Methylsterase
PROTEIN ACTIVITY	100	11	53	100	12	Methyltransferase
	100	1	50	100	6	Methyl-accepting activity

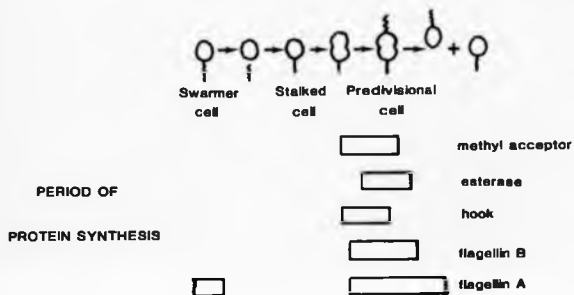


Fig. 1.10 Diagram of the *Caulobacter crescentus* cell cycle showing the periods of synthesis of specific proteins and the activity, in each cell type, of specific proteins, given as the percentage of the swarmer cell activity. From Gomes and Shapiro (1984).

to be more disparate than those of *E. coli* and *S. typhimurium*, which are clustered in three main regions.

Transposon mutagenesis of *C. crescentus* was attempted by Ely and Croft (1982), using the transposons Tn5 and Tn7. They found that Tn7 has a high degree of insertion site specificity in *C. crescentus*, and thus is not useful as a mutagen. However, when Tn5, which encodes a kanamycin resistance determinant, was introduced into *C. crescentus* on a plasmid which is not maintained in the bacterium, and several thousand kanamycin resistant clones were screened for auxotrophy and motility, approximately 1% were auxotrophic, and approximately 2% had altered motility. Thus it was concluded that Tn5 can insert in a large number of chromosomal sites in *C. crescentus*, and that it is a useful mutagen. The motility mutants have been important for studying control of expression of *fla* genes, as described later in this section. For this reason it was necessary to characterise and map the various mutations. This was done using generalised transduction and conjugation, with the Tn5 encoded kanamycin resistance providing a selectable marker (Barrett *et al.*, 1982). There is a partial genetic map showing the positions of the known *fla*, *mot* and *che* genes in Fig. 1.12, and a more detailed one with respect to other genes on the *C. crescentus* chromosome appears in Ely *et al.* (1986).

By 1982, the first flagellin genes from *C. crescentus* had been cloned. Milhausen *et al.* (1982) identified and cloned flagellin genes using anti-flagellin antibody. This was done by immunoprecipitation of polyribosomes from predivisional cells synthesising flagellin, followed by synthesis of a cDNA from the specifically precipitated mRNA. The cDNA was used as a probe to identify flagellin gene sequences from a

library of *C. crescentus* chromosomal DNA by hybridisation. In this way, the gene for the 29k flagellin, and part of the gene for the 25k flagellin were cloned. This was confirmed by comparing the previously established NH<sub>2</sub>-terminal amino acid sequence of the proteins with the clones. The immunoprecipitation of polyribosomes is an unusual method of cloning prokaryotic genes. It is more commonly used with eukaryotic mRNAs, which have a half-life of the order of hours, and is made more difficult with prokaryotic mRNAs as their half-life is of the order of 2-6 minutes.

A gene cluster specifying several *fla* genes, including structural genes for the 29k flagellin, the 27.5k flagellin and the 25k flagellin was cloned by Purucker *et al.* (1982). It was done by firstly cloning the Tn5 insertion, with some flanking *C. crescentus* chromosomal DNA, from a Tn5-generated non-motile mutant carrying the inserted transposon in the gene cluster mentioned above. This was done using the Tn5 encoded kanamycin resistance as a selectable marker. A portion of the flanking *C. crescentus* DNA was labelled with <sup>32</sup>P, and used to isolate the gene cluster from a library of wild type *C. crescentus* DNA by hybridisation. This gene cluster contained the fragments cloned by Milhausen *et al.*, plus some *fla* genes which appeared to have a regulatory function. Other genes for the 25k flagellin have been found elsewhere on the chromosome (Gill and Agabian, 1983).

The hook protein gene from *Caulobacter crescentus* was cloned by antibody screening of a  $\lambda$  library, using <sup>125</sup>I-labelled antibody. The library was prepared by partial digestion of *C. crescentus* chromosomal DNA with Sau3A. Fragments of 5-20Kb were inserted into the *Bam*HI site of the  $\lambda$  vector, generating a library of *C. crescentus* DNA with a high degree of

randomness. Several clones expressing the 70k hook protein were subsequently isolated. It has been shown that the hook gene occupies a single location on the *C. crescentus* chromosome (Ohta *et al.*, 1982).

Hahnenberger and Shapiro (1987) cloned and characterised a gene cluster required for basal body formation, the *flbN*, *flaD*, *B*, *C*, *flbO*, *motC* gene cluster. Like the cloning of flagellin genes by Furucker *et al.* (1982), this was done by firstly cloning the Tn5 insertion plus some *C. crescentus* DNA, by selecting for fragments encoding kanamycin resistance. They found that mutations in one of the genes in this cluster, *flaD*, allowed synthesis and assembly of a partial basal body consisting of the rod, M ring, S ring and E ring. Evidence from this mutant and others suggests that the flagellum in *C. crescentus* is assembled sequentially, starting from the cell-proximal parts of the basal body, as it is in *E. coli* (Suzuki and Komeda, 1981).

#### 1.5.2.3 Construction and use of a *C. crescentus* promoter probe

Before recounting how these cloned fragments of DNA were used to advance knowledge of *Caulobacter* differentiation, it is necessary to describe the promoter probe Tn5-VB32, which has also been used to investigate control of gene expression in *C. crescentus*.

Tn5-VB32 was constructed from a Tn5 derivative lacking the entire neomycin phosphotransferase II (NPT II) gene and a large portion of the left hand side insertion sequence IS50L, while retaining its ability to transpose. A fragment of DNA containing the NPT II gene lacking its promoter region but retaining its translation initiation signal was inserted into this, as shown in Fig. 1.11. Only the 23 leftward most bases of IS50L are essential for transposition, and 53 of these are



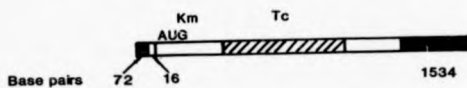


Fig. 1.11 Diagram of Tn5-VB32, promoter probe, from Bellofatto *et al.*, (1984). The black boxes are IS50 sequences, open boxes are unique sequences of Tn5 and the hatched box is the tetracycline resistance gene.

retained in Tn5-VB32. It was shown that insertion of Tn5-VB32 into the *C. crescentus* chromosome allows transcripts to initiate at a *C. crescentus* promoter, which then read through the remaining IS50L sequence into the intact, but promoterless, NPT II structural gene. IS50 contains a stop codon in all three reading frames within the first 30 bases, so it is not possible to generate hybrid proteins. Tn5-VB32 also contains a tetracycline resistance determinant as a selectable marker (Bellofatto et al., 1984).

The importance of Tn5-VB32 is that it allows the expression of NPT II to be controlled by *C. crescentus* flagellar promoters, and questions relating to the temporal regulation of flagellar genes can be addressed without the need to obtain purified protein products. NPT II synthesis can be measured by (i) determining the concentration of  $k_{cat}^{NPT II}$  which bacteria carrying a particular Tn5-VB32 insertion are resistant to and (ii) radioimmune precipitation using anti-NPT II antiserum. The latter method is more useful for measuring transcription from a promoter throughout the cell cycle.

Two drawbacks associated with promoter probes are (i) if there is control through autoregulation by the wild type protein product it will not be detected (Komeda and Iino, 1979) and (ii) different levels of expression can result from different fusions to the same promoter. This is probably because varying the number of base pairs or the DNA sequence between the *fla* promoter and the promoter probe insertion site can affect efficiency of transcriptional readthrough (Komeda, 1982). However, with controls, promoter probes are extremely valuable molecular biological tools for the study of microbial differentiation.

#### 1.5.2.4 Periodic synthesis of mRNA

Milhausen and Agabian (1983), using their cloned flagellin sequences in dot blot hybridisations, found that flagellin mRNA synthesis is regulated during the cell cycle. They isolated total RNA from synchronised *C. crescentus* cells every 10 minutes during the cell cycle. Each RNA sample was then immobilised on a nitrocellulose filter and excess  $^{32}\text{P}$  labelled flagellin DNA probe was allowed to hybridise to it. The amount of labelled probe hybridising to each sample was counted, and the temporal pattern of mRNA synthesis was found to coincide with that of expression of the flagellin polypeptides. The major period of mRNA synthesis occurred in the predivisive cell, and it was established that flagellin expression is controlled at the transcriptional level.

Ohta *et al.* (1985) using S1 nuclease mapping, with an internal fragment of the hook protein gene as a probe, showed directly that periodic expression of hook protein is regulated at the level of transcription. Total RNA isolated from synchronously developing *C. crescentus* cells at various times throughout the cell cycle was allowed to hybridise to excess  $^{32}\text{P}$  end-labelled probe, under conditions which favour formation of RNA DNA hybrids. Single stranded nucleic acid was digested with nuclease S1, and the mRNA bound, S1 resistant, labelled DNA was separated by gel electrophoresis and visualised by autoradiography. The experiment showed that the pattern of hook protein mRNA synthesis coincides with the periodic pattern of hook synthesis seen with radioimmune precipitation assays.

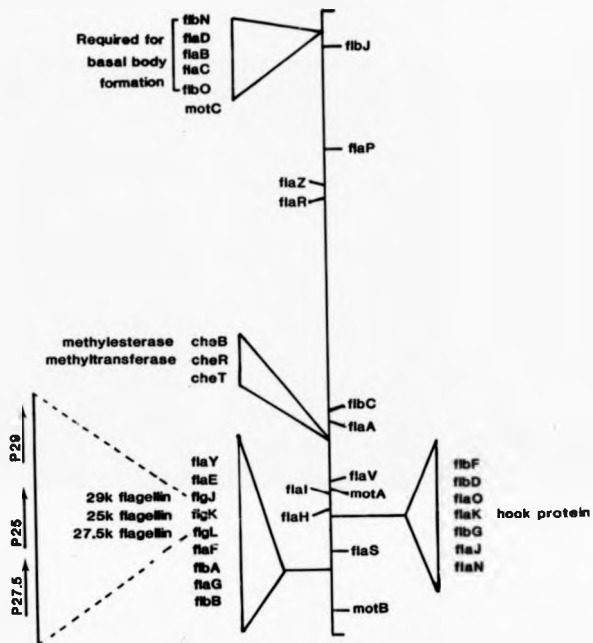
#### 1.5.2.5 The trans-acting hierarchy of flagellar and chemotaxis gene expression in *C. crescentus*

Experiments with cloned flagellar genes have been performed to

investigate the hypothesis that the chromosomal location of a gene controls when the corresponding protein is synthesised (Osley *et al.*, 1977 and section 1.5.1); and well characterised mutants, the cloned flagellar genes and Tn3-VB32 have been used to study the effect of expression of certain *fla* and *che* genes on the expression of other related genes.

The former experiments involved examining the influence of cloning flagellar genes on an independent replicon on the timing of expression. A strain of *C. crescentus* with an insertion mutation in the chromosomal hook protein gene, but carrying the wild type gene on an autonomously replicating plasmid, was constructed. The plasmid is replicated throughout the DNA synthesis phase. Radioimmune precipitation of hook protein from synchronised cells of this strain showed that the protein is synthesised with normal periodicity (Ohta *et al.*, 1984). The timing of duplication of this *fla* gene is not sufficient to determine the time at which it is synthesised in the cell cycle. Loewy *et al.* (1987) performed a similar experiment with *flgJ*, the gene for the 29k flagellin. They deleted it from the chromosome, and introduced a plasmid carrying the entire *flgJ* gene, including the 5' regulatory DNA, into the mutant strain. The time of initiation of synthesis of the 29k flagellin was the same as in wild type cells, showing that the gene does not have to be located on the chromosome for correct temporal control.

Bryan *et al.* (1984) used plasmid complementation to examine the *trans*-acting effect of certain cloned *fla* genes on other *fla* and *che* genes from *C. crescentus*. They constructed a plasmid containing the *flaY/E* region (Fig. 1.12) from wild type *C. crescentus*, and from this constructed two others which had deletions in *flaY*. The results of the



**Fig. 1.12** Partial genetic map of *Caulobacter crescentus* showing the location of *fla*, *che* and *mot* genes. Identified gene products are indicated. Modified From Bryan *et al.* (1987) and Minnich and Newton (1987).

complementation analysis were that *flaY* is required for the normal expression of the flagellin proteins, and motility. They found that mutations in the *flaY/E* cluster reduced levels of methyltransferase activity, and the membranes of such mutants had low levels of methyl-accepting ability. In the presence of the plasmid containing the intact *flaY/E* region methyltransferase activity was restored and methyl-accepting ability partially restored in these mutants. *flaE* is also required for expression of normal amounts of the flagellins and chemotaxis methylation functions (Bryan *et al.*, 1987).

As a consequence of the presence of cloned *flaY/E* genes on a plasmid being able to restore functions that are under the control of genes far apart on the chromosome, the authors suggested that a hierarchy of flagellar and chemotaxis expression may function as a control mechanism to coordinate the order of synthesis of specific proteins and the location of their assembly. Such a regulatory cascade controlling the expression of flagellar and chemotaxis genes has been demonstrated in *E. coli* (Komeda, 1982, 1986 and section 1.2.2).

Ohta *et al.* (1984) provided more evidence for a regulatory cascade by transducing Tn5 generated *fla* mutations in the hook gene cluster (Fig. 1.12) into other *fla* mutants. This showed that hook protein gene expression is required for synthesis of the flagellins, and that the expression of a number of other genes in the hook gene cluster is required for synthesis of the hook protein, and the flagellins. Again, a mutation in one *fla* gene had pleiotropic effects on the expression of unlinked *fla* genes, and the authors proposed that a regulatory cascade of trans-acting positive regulation factors exists in *C. crescentus*. In addition, it was found that mutations in the basal body genes described

in section 1.5.2.2 repressed expression of hook protein, and the 25k and 27.5k flagellins. Those which could not assemble a basal body at all also had very low levels of chemotaxis methylation, while the *flaD* mutant, which could assemble a partial basal body, exhibited normal levels of chemotaxis protein methylation. Mutants in *motC*, which assembles a flagellum, but are non motile, synthesised normal levels of all the flagellar structural proteins which were assayed (Hahnenberger and Shapiro, 1987). These data began to indicate the order of the cascade, and as in *E. coli*, it appears that genes required earlier in the construction of a flagellum, e.g. the basal body protein genes, are further back in the regulatory cascade than, for example, the flagellins, which are required late in the construction of the flagellum.

Double mutants containing one mutation in an assayable *fla* gene, and another *fla* gene with a Tr5-VB32 insertion allowed further deductions to be made about the regulatory cascade. Using such double mutants to ask if one *fla* gene alters the amount of transcription from another *fla* gene, Champer *et al.* (1987) were able to begin to group the known *C. crescentus* *fla* and *che* genes into regulons and partially order them within a hierarchy or regulatory cascade. In some *fla* mutants, synthesis of the flagellins, hook protein and chemotaxis methylation apparatus was reduced. In others, synthesis of flagellins and chemotaxis protein methylation machinery was reduced; while in others the synthesis of the flagellins and hook protein was reduced. Others synthesised greatly reduced amounts of the flagellins only. As yet, the gene at the beginning of the cascade, which has been identified in *E. coli* (Komeda, 1982, 1986) has not been identified in *C. crescentus*.

Thus, recent research indicates that in *C. crescentus* the control of the sequential assembly of a large number of proteins into the flagellar structure operates via a cascade control mechanism similar to that in *E. coli*, with the expression of genes further back in the cascade required for transcription (Minnich and Newton, 1987) of genes whose products are needed for the terminal stages in assembly of a flagellum.

However, evidence presented by Bryan *et al.* (1987) suggests that the temporal expression of the *fla* and *che* genes in *C. crescentus* requires an additional regulatory mechanism, as yet unknown. They found that temporal control of synthesis of flagellins and MCP methylating activity operated normally in *flaE* and *flaY* mutants synthesising much reduced, but just detectable, amounts of flagellins and chemotaxis methylation proteins. The genes are not located together on the chromosome, and the existence of a soluble messenger that can interact with them all to control temporal expression is a possibility.

#### 1.5.2.6 The role of specific DNA sequences in temporal control of gene expression

Information on the role of 5' regulatory sequences in the control of temporal expression comes from two sources: examination of the timing of NPT II expression driven by flagellar promoters, and identification of flagellar promoters by S1 mapping.

When genes which do have identified protein products, e.g. a flagellin gene, are accessed by Tn5-VB32, NPT II is synthesised with the same periodicity during the cell cycle as the flagellin. This is evidence that temporal control of gene expression occurs at the transcription level, and that the regulatory sequences that control



temporal expression reside within the 5' promoter regions of *C. crescentus* flagellar genes (Champer *et al.*, 1987).

As described in the previous section, Loewy *et al.* (1987) found that mating a plasmid containing the 29k flagellin gene into a strain deleted for the native gene resulted in the time of initiation of 29k flagellin expression remaining normal. Also, when the 29k flagellin promoter drove the reporter gene encoding NPT II, immunoprecipitation using anti-NPT II antibody showed that NPT II synthesis was initiated at the same time in the cell cycle as the synthesis of the 29k flagellin from the wild type gene. These findings indicate that the sequences 5' to the structural gene determine the time of initiation of expression of the 29k flagellin (Loewy *et al.*, 1987).

S1 mapping has proved very useful in identifying the location of *fla* promoters in *C. crescentus*. The extent of mRNA protection from nuclease S1 digestion of defined restriction fragments within cloned *fla* genes has allowed the identification of the promoters for 3 flagellin genes in the *flaEY* cluster (Minnich and Newton, 1987), the promoter for the hook operon and another promoter within the hook gene cluster (Chen *et al.*, 1986). In conjunction with DNA sequencing, the technique allows detailed study of the 5' regulatory sequences of *fla* genes. These promoters were compared, and conserved sequences were sought. Such sequences were found, and consensus sequences for the -13, -24 and approximately -100 regions were formulated. No homology to the *E. coli* -10 and -35 regions was found.

It was suggested that the *C. crescentus fla* conserved sequences could be involved in the binding of trans-acting positive regulatory factors to

the chromosome. The *C. crescentus* -13 and -24 consensus sequences bear homology to the -12, -24 regions of the *nif* promoter sequences reported for *Klebsiella pneumoniae* and a *Rhizobium* sp. The products of the nitrogen fixation genes in these bacteria are also positively regulated (Mullin *et al.*, 1987).

The work described, involving *E. coli*, *Bacillus subtilis*, *Myxococcus xanthus* and *Caulobacter crescentus* illustrates that pathways of dependence, or regulatory cascades, are a recurring feature in the control of timed gene expression during bacterial differentiation.

### 1.5.3 Control of positioning of cell components in *Caulobacter*

The predivisional cell of *C. crescentus* is a striking example of asymmetry within a single cell. How this structural and functional asymmetry is generated is a question of great interest and importance in the study of cellular differentiation. This dimorphic bacterium, which assembles a polar flagellum and stalk at specific sites in the cell envelope, and which divides by binary fission to yield two morphologically and physiologically distinct daughter cells provides an opportunity to examine how polarity within a cell is brought about.

*C. crescentus* cells do not form a septum before division, so the predivisional cell after DNA replication is a single cell containing the chromosomes of two different cell types. It has been suggested that mRNA encoding a protein destined for, say, the swarmer cell, could be localised as a result of differential transcription from only one of the chromosomes found in the predivisional cell (Milhausen and Agabian, 1983). Evinger and Agabian (1977) found that chromosome structure in swarmer cells, where no DNA replication is occurring, and stalked cells,

where DNA synthesis does take place, is different. Swarmer cell envelope associated nucleoids have a much higher sedimentation rate in sucrose gradients than stalked cell envelope-associated nucleoids, suggesting they are in a more condensed state. It is possible that changes in chromosome structure during differentiation could effect selective transcription. Evinger and Agabian also showed, in a 1979 paper, that quantitative and qualitative differences in the proteins isolated from swarmer cell envelope-associated nucleoids and stalked cell envelope-associated nucleoids do exist. These proteins are primarily derived from membrane.

Purucker *et al.* (1982) refer to the hypothesis that the location of a gene on the chromosome with respect to its physical relationship with the cell membrane may direct the cellular location of proteins from newly transcribed genes, and suggest that cloning a specific gene on an independent replicon and examining the effect of this on the spatial distribution of the protein in the cell could test this hypothesis.

What follows is a summary of experimental results with some bearing on control of spatial differentiation in *C. crescentus*.

Milhausen and Agabian (1983) used cloned flagellin DNA sequences in dot blot hybridisation experiments to examine the spatial localisation of flagellin mRNAs during *C. crescentus* development. From a synchronously differentiating population of cells, samples were collected at the stalked stage and the predivisional stage. The remaining predivisional cells were allowed to divide, and the daughter swarmer and stalked cells were isolated by density gradient centrifugation. Total RNA was isolated from all the samples and flagellin mRNA was assayed using <sup>32</sup>P

labelled flagellin DNA probes.

The parent stalked cell sample had a low basal amount of radioactivity bound to it, and the predivisional cell contained a greatly increased quantity of flagellin mRNA. The daughter stalked cell sample also had the low basal amount of radioactive DNA hybridising to mRNA, and the daughter swarmer cell sample contained more flagellin mRNA than the predivisional cell sample. These results indicated that the asymmetric expression of the flagellins is mediated through compartmentation of flagellin mRNA in the swarmer cell, i.e. flagellin mRNA is segregated into the swarmer cell at cell division. This is in agreement with the results of Osley *et al.* (1977), who found that *de novo* mRNA synthesis is not necessary for flagellin synthesis in the swarmer cell.

Gill and Agabian (1983) sequenced the 29k flagellin gene, and one of their findings was that there is no hydrophobic signal sequence at the NH<sub>2</sub>-terminus of the nascent protein. When the hook protein gene was sequenced it was noted that hook protein is synthesised as the mature protein, i.e. it also lacks a cleaved signal sequence (Ohta *et al.*, 1984). Thus these proteins are not targeted to membrane as they are synthesised, in the way that many bacterial membrane and periplasmic space proteins are. It has been suggested that flagellins and hook protein monomers are exported through a special channel formed by other polypeptide components of the flagellar organelle.

Another example of segregation of macromolecules to the swarmer cell at cell division was discovered when Gomes and Shapiro (1984) researched the question of the cellular distribution of the MCPs. The evidence for this can be seen in Fig. 1.10. In the daughter swarmer and stalked

cells there was little or no synthesis of MCPs or methylesterase, but maximum MCP methylation activity and methylesterase activity were found in the daughter swarmer cells. Therefore, the MCPs, methyltransferase and methylesterase are synthesised in the predivisional cell and segregated into the daughter swarmer upon cell division. The machinery to respond to chemotactic signals, like the flagellins, is positionally regulated within the predivisional cell.

#### 1.5.3.1 Isolation of flagellated membrane vesicles from *C. crescentus*

Treatment of *C. crescentus* cells with lysozyme and EDTA in the absence of sucrose results in cell lysis and the generation of a homogeneous population of vesicles that retain flagella. Incubating the lysate with an excess of anti-flagellin antibody and applying it to a Protein A-sapharose column allows separation of flagellated and non-flagellated vesicles. Flagellated membrane vesicles adsorb to the column; non-flagellated vesicles can be washed off (Huguenel and Newton, 1984a). This technique has been important in studying localization of membrane proteins in swarmer and predivisional cells.

Two dimensional polyacrylamide gel electrophoresis showed that a number of proteins were present predominantly or only in polar membranes (from flagellated vesicles), including flagellins A and B. Another set of proteins was found uniquely in the non-polar membrane fraction (Huguenel and Newton, 1984a).

Huguenel and Newton (1984b) reported the existence of three pools of flagellin in *C. crescentus*: soluble, membrane associated and assembled, the soluble and membrane pools being precursors in flagellum assembly. When vesicles are prepared from predivisional cells, the membrane pool

of flagellin is found in polar vesicles, but not in the non-polar vesicles. It was concluded from this that the asymmetric localisation of the flagellin pool is established at the flagellated cell pole before cell division (Huguenel and Newton, 1984a). The nature of membrane associated flagellin in *C. crescentus* is not known, but it does not appear to be an integral membrane protein. It is possible that the flagellar subunits are translocated to the cell surface for assembly via the basal structure of the flagellum without direct insertion into the membrane (Huguenel and Newton, 1984a).

The fact that MCPs can be identified by labelling with [methyl-<sup>3</sup>H] methionine *in vivo* and *in vitro* (Shaw *et al.*, 1983) was used by Nathan *et al.* (1986) to examine the differential localisation of MCPs in predivisinal and swarmer cells of *C. crescentus*. They used the separation procedure described above to separate flagellated and non-flagellated vesicles. Additionally, they found that, with the use of different lysis buffers, it could be arranged that small vesicles (around 10% of swarmer cell volume) or large vesicles (50-70% of swarmer cell volume) were formed.

With small vesicles from swarmer cells, tritiated MCPs were detected in flagellated and non-flagellated vesicles, indicating that MCPs in newly divided swarmer cells are present over the entire cell, rather than being clustered at the flagellated cell pole.

With large vesicles from pre-divisinal cells, tritiated MCPs were located almost exclusively in the flagellated vesicles, showing that localisation of chemotaxis proteins to the swarmer cell portion of the predivisinal cell occurs before cell division, and corroborating the

findings of Gomes and Shapiro (1984).

Densitometry scans of autoradiograms of gels containing tritiated MCPs from small vesicles from swarmer cells do show slightly higher amounts of MCPs in flagellate vesicles compared with non-flagellate vesicles, and it has been suggested that a gradient of MCPs over the predivisional cell with the highest concentration near the flagellate pole, could exist (Nathan *et al.*, 1986).

#### 1.5.3.2 Some recent data on localisation of macromolecules in *C. crescentus*

Rauter and Shapiro (1987) identified four major heat shock proteins in *C. crescentus*, predominantly through cross-reactivity with antisera to the *E. coli* heat shock proteins.

Their reason for looking for heat shock proteins in *C. crescentus* was that in order to investigate more effectively the mechanisms that regulate asymmetric segregation and cell-specific gene expression, it is desirable to identify proteins that segregate to the stalked cell. (All four of the heat shock proteins were synthesised in the predivisional cell.) They found that three of the heat shock proteins, the 92k Lon homologue, the 70k DnaK homologue and the 37k RNA polymerase subunit, under both normal and stress conditions, segregate to the replication-competent stalked cell. One heat shock protein, the 62k GroEL homologue, segregated to both swarmer and stalked cell upon division. The authors suggested that the functional significance of the segregation of 3 of these proteins to the stalked cell is connected with the fact that the stalked cell can immediately undergo another round of DNA synthesis and cell division, unlike the swarmer, which must first

shed its flagellum and synthesise a stalk.

It is hoped that cloning these genes will shed more light on how mRNA localisation is brought about. There are three possibilities for differential mRNA localisation in one of the two cell types after division. These are:

- (i) sequestering of specific prädivisional cell mRNA into the correct recipient cell.
- (ii) that classes of specific mRNA molecules not required in one of the cell types are rapidly degraded in that cell type, and are significantly more stable in the correct recipient cell type.
- (iii) specific mRNAs are only transcribed from the chromosome in the correct half of the prädivisional cell, and remain there until after cell division.

Future experiments should reveal which of these possible mechanisms operate in *C. crescentus* differentiation.

The question of whether the structure of the sequestered protein is important in localisation has been addressed by Loewy *et al.* (1987). Having established that the 5' regulatory region of the 29k flagellin gene is involved in the control of temporal expression, they asked if the 5' region of a gene encoding a protein contributes to its correct positioning. Using the 29k flagellin promoter (5' regulatory region) fused to the NPT II structural gene they assayed where the NPT II synthesised from the fusion was found in daughter swarmer and stalked cells. NPT II was not segregated to the swarmer, as the 29k flagellin was, but was found predominantly in the stalked cell. This segregation pattern was the same as that for NPT II driven by its own promoter. These results imply that the nucleotide sequences responsible for



differential expression of the 29k flagellin do not contribute to the positioning of the protein within the cell, but that the protein coding region is necessary for localisation.

Loewy *et al.* also investigated whether flagellum assembly is necessary for correct localisation of the 29k flagellin. In the absence of flagellum assembly in a hook protein mutant, the 29k flagellin still segregated to the swarmer progeny cell.

The last decade has seen many important advances in the study of control of *Caulobacter* differentiation, and as a consequence of these, a greater understanding of these processes of bacterial morphogenesis can be anticipated.

#### 1.6 Differentiation in *Rhodomicrobium vannielii*

*Rhodomicrobium vannielii* is a member of the *Rhodospirillaceae* (purple non-sulphur bacteria) which can grow either photoheterotrophically or microaerophilically in the dark (section 2.4). It divides by budding, i.e. it has only one active, polar growth point per cell at any one time (Whittenbury and Dow, 1977), and this obligate polar growth pattern results in asymmetry of structure and function in predivisional cells (Kelly and Dow, 1984).

Three types of cell are present in batch culture of *Rm. vannielii*: complexes of cells linked by filaments (multicellular arrays), unattached motile cells (swarmers) and non-motile angular cells (exospores). In order to determine the sequence of events leading to

the formation of these cell types, synchronised swarmer cells were prepared, which give rise to all the other morphological types observed (Whittenbury and Dow, 1977). The studies on synchronised swarmer cells established that *Rm. vannielii* has two vegetative cell cycles, expressed according to specific environmental conditions (which can be controlled in the laboratory), and exospores can be formed which have increased resistance properties to desiccation, heat and ultra-violet irradiation.

When synchronised swarmer cells are incubated anaerobically in the light, in the presence of low  $\text{CO}_2$  concentrations, the peritrichous flagella are shed, a filament or stalk is synthesised at one pole of the cell, and synthesis of a bud begins. The daughter cell grows to the same size as the mother cell, and physiological separation of the two cells is achieved by the synthesis of a plug within the filament a short distance from the daughter cell. The plug can be seen clearly in the electron microscope. The whole process takes approximately six hours. It is this obligate sequence of differentiation events (shown in more detail in Fig. 1.14), which has been examined during the course of this study, and which is referred to as the *Rm. vannielii* cell cycle.

Under the conditions described (high light intensity and low  $\text{CO}_2$  concentration) a multicellular array will be formed (see Fig. 1.13). However, under conditions of low light intensity and high  $\text{CO}_2$  concentration, the second vegetative cell cycle, known as the simplified cell cycle, is expressed. This is also shown in Fig. 1.13, and consists of constitutive swarmer cell production from a non-motile, stalked reproductive cell. Cell separation is exclusively by binary fission, and the formation of multicellular arrays is excluded (Dow and France, 1980).

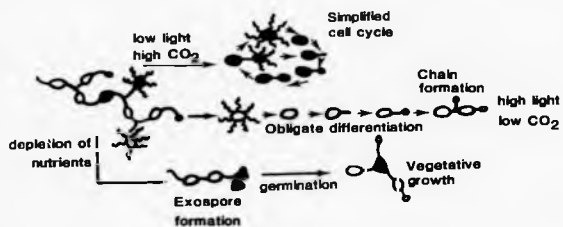


Fig. 1.13 Cell expression in *Rhodospirillum rubrum* (from Dow et al., 1983).

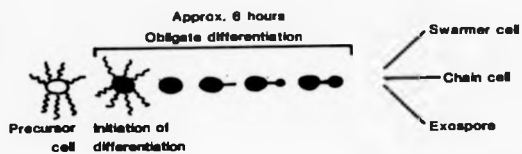


Fig. 1.14 The *Rhodomicrobium vannielii* swarmer cell cycle (from Dow et al., 1983).

like *C. crescentus* swarmer cells, *Rm. vannielii* swarmer cells are not capable of reproduction until they have shed their flagella and synthesised a stalk. There are other similarities between swarmer cells of *Caulobacter*, *Rhodomicrobium* and the chemoheterotrophic budding bacterium *Hyphomicrobium*, and the function of the swarmer cells of these bacteria in nature is the subject of discussion by Dow *et al.* (1983) and Kelly and Dow (1984), drawing on experimental results obtained in the laboratory.

#### 1.6.1 *Rhodomicrobium vannielii* as a system for the study of microbial differentiation

The cell cycles of *Rm. vannielii* provide an interesting opportunity to study morphogenesis and differentiation in a photosynthetic bacterium.

In *Rhodomicrobium*, the photosynthetic apparatus is located in a system of lamellate intra-cytoplasmic membranes (ICM). The synthesis of these photosynthetic membranes is one differentiation event which has been studied during the cell cycle. As a consequence of the polar growth process, the ICM complex must be formed *de novo* in the daughter cell during differentiation, involving temporal and spatial control of gene expression (Kelly and Dow, 1986). As in other photosynthetic bacteria, ICM content can be controlled by varying environmental conditions. If the bacteria are grown at high light intensity, only one double layer of membrane (in addition to the cytoplasmic membrane) can be observed by electron microscopy of thin sections. If the bacteria are grown at low light intensity, they are packed with multiple layers of ICM (Whittenbury and Dow, 1977).

*Rm. vannielii* meets the criteria for a bacterial model system for cellular development laid down by Shapiro *et al.* (1971). Swarmer cells incubated anaerobically in the light develop through an obligate series of well defined morphological stages, and large quantities of synchronised swarmer cells can be obtained by selective filtration (Whittenbury and Dow, 1977). Between 100 ml and 15 l cultures of *Rm. vannielii* can be synchronised by filtration through a glass wool column (section 2.6), to give a homogeneous population of swarmer cells containing around  $2 \times 10^7$  viable cells  $\text{ml}^{-1}$ .

A further advantage of *Rm. vannielii* is that swarmer cells held anaerobically in the dark fail to differentiate, until exposure to light triggers them to embark on differentiation synchronously. Therefore, as it is possible to separate energy source and carbon source, comparison of biochemical and morphological events in non-differentiating and differentiating cells can be made, and it can be shown that observed biochemical changes in differentiating cells from the same culture, treated in the same way as non-differentiating cells, are a consequence of light-induced differentiation.

The distinction between differentiation triggered by an environmental cue, such as nutrient depletion in *Bacillus subtilis*, and differentiation which is an obligate part of the cell cycle, as in *C. crescentus*, becomes blurred when *Rm. vannielii* is studied. It appears to fall into the latter category, but because it is possible to hold swarmer cells (which remain viable for more than 16 hours), anaerobically in the dark, it can be seen that this differentiation is very much controlled by an environmental factor, the availability of light.

Although it is not possible to separate the energy source and carbon source with *C. crescentus*, there is evidence that swarmer cells accumulate as the late exponential phase is reached. Variation in aeration and temperature regimes in several culture media have yielded similar results (Swoboda, 1979). These data have been taken by Dow *et al.* (1983) to indicate that *C. crescentus*, like *Rm. vannielii*, possesses a regulatory mechanism which ensures that the swarmer cell does not initiate differentiation unless environmental conditions are conducive to completion of the cell cycle.

It is important that bacteria used for differentiation studies grow on defined media, to allow the correlation of biochemical events with morphogenesis and differentiation. As described in section 2.2, *Rm. vannielii* grows in a simple pyruvate, malate and mineral salts medium.

Also, the availability of mutants and a system for the exchange of genetic information are useful for the study of differentiation in a bacterium. In the course of research carried out contemporarily with this study, it has been shown that *Rm. vannielii* can be effectively mutagenised with ultra-violet radiation, N-methyl-N'-nitro-N-nitrosoguanidine and the transposon Tn5 (sections 2.42 and 5.3) that it is possible to transfer genetic information from *E. coli* into *Rm. vannielii* by conjugation (R. L. Breadon, C. S. Dow and G. P. C. Salmond, unpublished).

The following is a brief review of work done on differentiation in *Rm. vannielii*.

### 1.6.2 Experimental data on differentiation in *Bm. vannielli*

#### 1.6.2.1 Synthesis of proteins in synchronised populations of

##### *Bm. vannielli*

Protein synthesis during the cell cycle was assayed in synchronised *Bm. vannielli* populations by determining the incorporation of tritiated leucine into cellular material. This increased steadily during the first three hours, then the rate of incorporation slowed down for approximately one hour, corresponding to the onset of filament formation. When this was complete, the rate of incorporation increased steadily (Whittenbury and Dow, 1977).

When synchronised cells were labelled with 10 minute pulses of [ $^{35}$ S]methionine at hourly intervals during differentiation, it was revealed that there are quantitative and qualitative changes in protein synthesis during differentiation, compared with protein synthesis in non-differentiating swarmer cells (Porter, 1984; Porter and Dow, 1987).

Studies on protein synthesis in synchronised swarmer cells incubated under different regimes of light, dark and aerobicity showed that one soluble protein of molecular weight 11.5k is synthesised to a much greater extent when cells are held anaerobically in the dark for 6 hours, compared with cells incubated anaerobically in the light for 6 hours. The 11.5k protein is only synthesised in swarmer cells incubated anaerobically in the dark, and is degraded if the culture is illuminated, or exposed to air in the dark. Porter and Dow (1987) suggested that the protein may have a role in the inhibition of differentiation of swarmer cells under conditions of light limitation, i.e. energy limitation.

Kelly and Dow (1986) examined temporal changes in intra-cytoplasmic



membrane (ICM) protein synthesis during the *Rm. vannielii* cell cycle. Samples taken from a synchronised culture were pulse labelled at one hour intervals with [ $^{35}$ S]methionine, and ICMs were prepared from each sample by centrifugation on to a sucrose cushion. Polyacrylamide gel electrophoresis of the samples showed that there is a temporal programme of ICM protein synthesis in *Rm. vannielii*. In addition, labelling cells in a batch culture, then separating swarmer cells and multicellular arrays showed that there are differences in the ICM proteins synthesised by the two cell types.

Among the proteins whose rate of synthesis was found to be regulated during differentiation were a 34k protein, thought to be flagellin, which was only synthesised in swarmer cells, and a polypeptide of 14k which was not synthesised, or only at very low rates, in swarmer cells, but whose synthesis increased from 3 hours into differentiation to completion of daughter cell formation. This protein was identified as a component of the reaction centre-associated light-harvesting complex I (LHI, Kelly and Dow, 1985) and is particularly strongly synthesised during daughter cell formation. Kelly and Dow (1986) point out that this is consistent with the proposal that new photosynthetic units are incorporated into the daughter cell membrane during differentiation as a consequence of polar growth.

The synthesis of a group of proteins of 70-80k molecular weight could be detected only in multicellular arrays, but the presence of these proteins in swarmer cells was detected on silver stained gels. This led to the suggestion that these may be an example of proteins synthesised in the reproductive cells and segregated to the swarmer cell upon division (Kelly and Dow, 1986), as in *C. crescentus* (section 1.5.3.2).

Some work on protein phosphorylation in *Rm. vannielii* has been carried out by Turner and Mann (1986). Protein phosphorylation has now been demonstrated in a wide range of bacteria, and its regulatory role in isocitrate dehydrogenase activity in *E. coli* has been characterised (Nimmo, 1984). In *Rm. vannielii* at least 25 proteins are phosphorylated.

Pulse labelling of a synchronised population of differentiating *Rm. vannielii* cells with [ $^{32}$ P]orthophosphate resulted in the finding that phosphorylated proteins are not detectable in the swarmer cell prior to differentiation but, as differentiation proceeds, a 55k phosphoprotein and an 86k phosphoprotein become increasingly abundant, and several minor phosphorylated species appear. Additionally, pulse labelling during growth of a batch culture showed that at least three proteins show a growth stage-specific pattern of phosphorylation. One of these is the aforementioned 55k protein, the phosphorylated species becoming increasingly abundant during later stages of growth (Turner and Mann, 1986).

#### 1.6.2.2 RNA synthesis

RNA synthesis in a synchronised population of swarmer cells developing anaerobically in the light was examined by Potts and Dow (1979). They found that while RNA synthesis does take place in the swarmer cell, there is a large increase in RNA synthesis 3 hours into differentiation, corresponding to the onset of daughter cell synthesis.

Pulse labelling experiments have shown that there is no rRNA synthesis in the swarmer cell, and that synthesis begins at the initiation of differentiation (Dow *et al.*, 1985), which has been classed as more

evidence that the swarmer cell is essentially a growth precursor cell where net biomass increase does not occur.

It was the fact that there is a significant change in the rifampicin sensitivity of synchronised *Rm. vannielii* cells during the cell cycle that prompted Scott and Dow (1986a) to purify and partially characterise the RNA polymerase of *Rm. vannielii*. Reproductive cells are sensitive to lower concentrations of rifampicin than swarmer cells (Whittenbury and Dow, 1977), which raised the possibility that RNA polymerase modification may occur during differentiation. RNA polymerase from swarmer cells and RNA polymerase from reproductive cells were isolated, and their subunit structures were compared on silver-stained one-dimensional and two-dimensional polyacrylamide gels (Scott and Dow, 1986b, Scott *et al.*, 1987). The enzyme composition is similar in both cell types, in that  $\beta$ ,  $\beta'$ ,  $\alpha$  and  $\sigma$  subunits with the same molecular weights are seen on gels. Amounts of minor proteins associated with the enzyme do change during differentiation, but it is not known if they are concerned with RNA polymerase function (Scott *et al.*, 1987).

However, one explanation for the differing rifampicin sensitivities of swarmer cells and reproductive cells is that the swarmer cell envelope is less permeable to hydrophobic inhibitors than that of the mother cell. This is discussed below.

#### 1.6.2.3 The cell envelope of *Rm. vannielii*

The lines of evidence which led Scott *et al.* (1987) to propose that there may be a change in cell envelope permeability properties during differentiation of *Rm. vannielii* swarmer cells included (1) that the swarmer cell is more resistant to rifampicin than the reproductive cell

(Whittenbury and Dow, 1977; Scott *et al.*, 1987), (ii) that the swarmer cell is less permeable to nucleotides (Scott and Dow, 1986b) and (iii) that the swarmer cell is more resistant to detergent lysis than the reproductive cell (Scott *et al.*, 1987).

Like the other purple photosynthetic bacteria, *Rm. vannielii* is Gram negative.

Recently, the lipopolysaccharide (LPS, Holst *et al.*, 1986) and peptidoglycan (Jürgens *et al.*, in press) from swarmer cells and reproductive cells of *Rm. vannielii* strain Rm5 have been isolated and compared. No qualitative and essentially no quantitative differences in sugar and fatty acid composition of LPS from swarmer and chain cells were observed (Holst *et al.*, 1986).

When peptidoglycan was isolated from swarmer cells and chain cells, no differences in peptidoglycan composition, degree of cross linkage or proteins bound to peptidoglycan were found. However, it was found that peptidoglycan from *Rm. vannielii* has a 10-30% lack of N-acetylation of glucosamine, which explains why *Rm. vannielii* cells are less sensitive to lysozyme (Jürgens *et al.*, in press).

Thus, the permeability differences between swarmer and reproductive cells cannot be explained by differences in peptidoglycan or LPS structure.

In contrast to rifampicin sensitivity, chloramphenicol sensitivity did not vary during the cell cycle (Scott *et al.*, 1987). Rifampicin is a hydrophobic inhibitor, and these molecules do not permeate through porin

channels in enteric bacteria, but through the lipid bilayer (Nikaido and Vaara, 1985). It is thought that chloramphenicol may enter *Rm. vannielii* through porin channels (Scott *et al.*, 1987), which suggests that porin channels do not change during differentiation, and that the observed permeability changes are more likely membrane associated. Porins from *Rm. vannielii* have not been identified, but a porin protein has been isolated and characterised from the related bacteria *Rhodobacter capsulatus* (Flammann and Weckesser, 1984) and *Rhodobacter sphaeroides* (Weckesser *et al.*, 1984).

It has been shown by Neunlist *et al.* (1985) that *Rm. vannielii* is a good source of bacteriohopanes, which are thought to act as membrane reinforcers as sterols do in the membranes of eukaryotes. The precise location of these triterpenoid molecules in the cell envelope is not known, but they might affect permeability. Kelly (1985) raises the possibility that *Rm. vannielii* may have a protein surface array outside the outer membrane, which could change during differentiation.

Although the structural changes underlying the differences in permeability between swarmer cells and reproductive cells of *Rm. vannielii* are not understood, it is not unlikely that these two cell types with different functional and ecological roles could have cell envelopes with differing structure and functions.

#### 1.6.2.4 The *Rm. vannielii* chromosome and DNA synthesis

The genome of *Rm. vannielii* was studied by Potts *et al.* (1980). The DNA was found to be approximately two-thirds of the *E. coli* genome in size. No plasmid DNA was detected.

Chromosomal DNA was isolated from swarmer cells, multicellular arrays and cells from heterogeneous cultures growing via the simplified cell cycle. There was no difference in the melting curves obtained with the three DNA samples, and the reassociation kinetics were also exactly the same. Comparison of the reassociation kinetics of *Rm. vannielii* DNA with those of *E. coli* DNA, however, showed that about 5% of the *Rm. vannielii* DNA is present as a rapidly renaturing component, which was present in all cell types examined. Electron microscopy of heat-denatured, rapidly cooled DNA suggested that the rapidly renaturing component consisted of short inverted repeats of approximately 400 base pairs in length (Potts *et al.*, 1980). Their function is unknown, though several suggestions have been made (Potts *et al.*, 1980, Russell and Mann, 1986).

Inverted repeat DNA in *Rm. vannielii* was further analysed by examining nuclease S1 resistant fragments of denatured, then rapidly renatured, chromosomal DNA (Russell and Mann, 1986). They found that approximately 7% of *Rm. vannielii* chromosomal DNA is inverted repeat DNA, and that it is present in two size classes. The large inverted repeat DNA was heterogeneous and contained species in the size range 100 bp - 700 bp, while the smaller size class contained species of 17 and 27 bp. These sequences are dispersed throughout the genome.

DNA was prepared from heterogeneous cell populations, undifferentiated swarmer cells and reproductive cells. When these DNA samples were restricted, and probed with both size classes of inverted repeat DNA in Southern blots, there were no distinct differences in hybridisation pattern between the different differentiation stage-specific DNAs. This was taken to mean that inverted repeat DNA sequences are probably not

involved in major cell cycle associated DNA rearrangements (Russell and Mann, 1986).

Microscopic studies of differentiating swarmer cells suggest that the conformation of the *Rm. vannielii* nucleoid varies during the cell cycle; being highly condensed in the swarmer cell, and larger in size and more dispersed in the reproductive cell. This is born out by the sedimentation values of envelope associated nucleoids (EANs) from the two cell types. The swarmer cell EAN has a higher sedimentation value than that of the reproductive cell, indicative of a more condensed conformation (Dow *et al.*, 1985). These results are identical to those found with *C. crescentus* (Evinger and Agabian, 1977). Also, as in *C. crescentus* (Evinger and Agabian, 1979) there are characteristic qualitative and quantitative differences in the polyacrylamide gel profiles of the DNA binding proteins from swarmer cells and reproductive cells (Dow *et al.*, 1985).

In a population of synchronised *Rm. vannielii* cells incubated anaerobically in the light, DNA synthesis is not detectable in the swarmer cell, but is initiated immediately after prostheca formation (Potts and Dow, 1979). This closely parallels the situation in *C. crescentus*. In dark incubated cells there is little DNA synthesis, which adds to the weight of evidence that, in nature, the role of the swarmer cell is one of dispersal (Potts and Dow, 1979).

Section 1.6 has aimed to provide a short review of what is known of differentiation in *Rhodomicrobium vannielii*, in order to allow the results presented in this study to be interpreted in the context of other data. Several striking similarities in (1) the swarmer cell

cycles and (ii) the differing properties of swarmer cells and reproductive cells in *Caulobacter* and *Rhodospirillum rubrum* are apparent from this introduction, and they are more fully discussed elsewhere (Now et al., 1983).

#### 1.6.3 Summary

To recap, the reasons for studying *Rm. vannielii* rather than *C. crescentus* are:

1. Much larger numbers of synchronised swarmer cells can be obtained by a simple, rapid procedure.
2. It is possible to control whether these cells remain motile growth precursor cells or differentiate into stalked reproductive cells simply by incubation in light or dark.
3. The obligate polar growth mode, in addition to the ease of obtaining synchronised cells, enable the study of the assembly of new photosynthetic units in *Rm. vannielii*.

The following is a list of the differing properties of swarmer and stalked cells documented so far:



## SWARMER

## STALKED CELL

Motile, non-prosthecae

Non-motile

Has dispersal function, and  
is not capable of reproduction

Is capable of reproduction

Biomass of cell does not  
increase

Growth occurs

More resistant to rifampicin  
and detergent lysis

More sensitive to rifampicin  
and detergent lysis

No DNA synthesis

DNA synthesis is initiated after  
prosthecae formation

No rRNA synthesis

rRNA synthesis occurs

Condensed nucleoid conformation

Larger, dispersed nucleoid

Phosphoproteins not detected

Phosphoproteins detected

Different intracytoplasmic membrane proteins and DNA binding proteins  
are synthesized by the two cell types.

## CHAPTER 2

## MATERIALS AND METHODS

## 2. MATERIALS AND METHODS

### 2.1 Organisms

Table 2.1 lists the bacteria used in this study, and Table 2.2 details some genetic characteristics of the *E. coli* strains used.

### 2.2 Media

*Rhodocrobium vannielii* was grown on pyruvate-malate medium (PM, Whittenbury and Dow, 1977) which contained per litre:

0.5 g	ammonium chloride	( $\text{NH}_4\text{Cl}$ )
0.4 g	magnesium sulphate	( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ )
0.4 g	sodium chloride	( $\text{NaCl}$ )
0.05 g	calcium chloride	( $\text{CaCl}_2$ )
1.5 g	sodium pyruvate	
1.5 g	sodium hydrogen malate	

The pH of the solution was adjusted to 6.8 - 6.9 with potassium hydroxide (KOH) pellets. The medium was autoclaved at 121°C for 15 minutes. After cooling, sterile phosphate buffer was added aseptically to a final concentration of 5 mM. (0.1 M phosphate buffer stock solution contained potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) 6.8 g l<sup>-1</sup> and dipotassium hydrogen phosphate ( $\text{K}_2\text{HPO}_4$ ) 8.7 g l<sup>-1</sup>, pH 6.8.)

The other members of the *Rhodospirillaceae* family were grown in medium

Table 2.1 Bacteria Used in This Study

	STRAIN	REFERENCE
Rhodomicrobium vannielii	Rm5	Whittenbury & Dow, 1977
Rhodomicrobium vannielii	RB2	
Rhodobacter capsulatus	NCIB 8254	Eckersley & Dow, 1980
Rhodobacter sphaeroides	NCIB 8253	
Rhodospseudomonas blastica	NCIB 11576	
Rhodospseudomonas acidophila	ATCC 25092	
Rhodospseudomonas palustris	NCIB 8288	
Rhodospseudomonas viridis	ATCC 19567	
Rhodocyclus galatinosus	NCIB 8290	Poindexter, 1964
Rhodospirillum rubrum	NCIB 8255	
Caulobacter crescentus	CB13	
Staphylococcus aureus	Cowan serotype 1 NCTC 8530	Hanahan, 1983 Young & Davis, 1983 Simon et al., 1983
Escherichia coli	DH1	
	Y1090	
	S17-1	

RB2 is a nalidixic acid resistant derivative of Rm5, kindly given to me by Robert Breadon of this laboratory. *C. crescentus* CB13 was a gift from Dr. David Hodgson of this laboratory.

Table 2.2 Genetic Characteristics of *E. coli* Strains Used in  
This Study

	CHARACTERISTICS	PLASMID PHENOTYPE	SOURCE
DH1	F <sup>-</sup> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> , ( <i>r<sub>k</sub></i> <sup>-</sup> <i>m<sub>k</sub></i> <sup>+</sup> ), <i>supE44</i> , <i>relA1?</i> , $\lambda$ <sup>-</sup> .		Neil Crickmore, this laboratory
Y1090 (x <sup>-</sup> )	<i>AlacU169</i> , <i>proA</i> <sup>+</sup> , <i>Alon</i> , <i>araD139</i> , <i>strA</i> , <i>supF</i> [ <i>trpC22::Tn10</i> ] <i>hsdR</i> <sup>-</sup> , <i>hsdM</i> <sup>+</sup> , (pMC9).	pMC9 is pBR322- <i>lacI</i> <sup>Q</sup>	Promega Biotec
S17-1	F <sup>-</sup> , <i>thi</i> , <i>pro</i> , ( <i>r<sub>k</sub></i> <sup>-</sup> <i>m<sub>k</sub></i> <sup>+</sup> ), ::RP4-2-Tc::Mu- Km::Tn7, (pSUP2021).	pSUP2021 carries Ap <sup>r</sup> , Cm <sup>r</sup> and has Tn5 inserted in its Tc resistance gene, rendering it Tc <sup>s</sup> , Km <sup>r</sup> .	Robert Breadon, this laboratory.

prepared in the same way, with the addition of 0.1% (w/v) yeast extract before autoclaving. This was known as PHV medium.

*Caulobacter crescentus* was grown in (i) a complex medium, and (ii) a defined medium, according to the needs of the experiment.

- (i) Peptone yeast extract medium (PYE) contained per litre:

Bacto peptone	2.0 g
Yeast extract	1.0 g
Magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ )	0.2 g

This was autoclaved at  $121^\circ\text{C}$  for 15 minutes.

- (ii) M2 medium (Contreras *et al.*, 1978) contained per litre:

$\text{Na}_2\text{HPO}_4$	1.74 g
$\text{KH}_2\text{PO}_4$	1.06 g
$\text{NH}_4\text{Cl}$	0.5 g

50 ml aliquots of this were autoclaved at  $121^\circ\text{C}$  for 15 minutes in 250 ml conical flasks.

The following stock solutions were prepared and autoclaved separately:

50 mM	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
30% (w/v)	glucose
50 mM	$\text{CaCl}_2$

10 mM  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was also prepared and filter sterilized. These four stock solutions were 100x the concentration required in the medium. The

appropriate amount of each was added aseptically to the sterile buffer in the culture flask.

*Staphylococcus aureus* was grown in nutrient broth (Oxoid) prepared according to the manufacturer's instructions.

*Escherichia coli* was grown in Luria Bertani medium (LB). This contained per litre:

Difco bacto tryptone	10 g
Yeast extract	5 g
NaCl	10 g

This was adjusted to pH 7.5 with sodium hydroxide (NaOH) pellets and autoclaved at 121°C for 15 minutes.

For solid media, 1.5% (w/v) Difco bacto agar was added routinely before autoclaving.

### 2.3 Antibiotics

To prepare stock solutions, antibiotic was dissolved in sterile double distilled water or alcohol as appropriate. Media were allowed to cool to around 50°C before antibiotics were added. Table 2.3 describes the use of the various antibiotics employed in this study.

Table 2.3 Antibiotics Used in This Study

ANTIBIOTIC	STOCK SOLUTION	FINAL CONC. IN MEDIA	STORAGE CONDITIONS
Ampicillin	25 mg ml <sup>-1</sup> of the sodium salt in water	50 µg ml <sup>-1</sup>	4°C or -20°C
Chloramphenicol	34 mg ml <sup>-1</sup> in ethanol	10 µg ml <sup>-1</sup>	-20°C
Cycloheximide	10 mg ml <sup>-1</sup> in water	50 µg ml <sup>-1</sup>	4°C
Kanamycin	25 mg ml <sup>-1</sup> of kanamycin sulphate in water	25 µg ml <sup>-1</sup>	4°C or -20°C
Nalidixic acid	10 mg ml <sup>-1</sup> in 30 mM NaOH	100 µg ml <sup>-1</sup>	4°C
Streptomycin	20 mg ml <sup>-1</sup> of streptomycin sulphate in water	25 µg ml <sup>-1</sup>	4°C or -20°C
Tetracycline	12.5 mg ml <sup>-1</sup> of tetracycline hydrochloride in 50% (v/v) ethanol/water	12.5 µg ml <sup>-1</sup>	-20°C in the dark



## 2.4 Growth

*Rhodospirillum rubrum* was grown (i) anaerobically in the light in liquid and on agar plates, and (ii) microaerophilically in the dark on agar plates.

(i) *Rm. rubrum* was grown in 250 ml Quickfit conical flasks sealed with rubber Suba seals. A 1 ml inoculum was used in 100 ml of medium, then each flask was flushed with oxygen-free nitrogen for 10 minutes through sterile syringe needles inserted in the Suba seals. Incubation was in a shaking water bath at 30°C with an incident light intensity of  $25 \mu\text{Em}^{-2}\text{s}^{-1}$  provided by tungsten lamps.

On a larger scale *Rm. rubrum* was grown in 5 litre or 20 litre flat-bottomed vessels (Baird and Tatlock) with Quickfit tops which could be sealed with Suba seals. After inoculation (0.3% v/v) these were flushed with oxygen-free nitrogen for 30 minutes, and incubated in a warm room at 30°C with an incident light intensity of  $35 \mu\text{Em}^{-2}\text{s}^{-1}$ . Cultures were stirred continuously by magnetic stirrers. A dense culture was achieved after 2.5 days growth.

Growth was followed by measuring the optical density (O.D.) of cultures at 540 nm in a Pye Unicam SP500 spectrophotometer.

Agar plates were incubated under anaerobic conditions in the light using the anaerobic bag technique described by Westmacott and Primrose (1975). Plates were placed on a tray within transparent nylon bags (Portex Ltd., Hythe, Kent) which were heat sealed and flushed with oxygen-free nitrogen for 15 minutes. A beaker containing saturated pyrogallol

solution (20 ml) and 15% (w/v) potassium carbonate, 10% (w/v) sodium hydroxide solution (10 ml) was also included in the bag to remove traces of oxygen. Incubation was at 30°C at a light intensity of  $35 \mu\text{Em}^{-2} \text{s}^{-1}$ .

(ii) It has recently been found by Robert Breadon of this laboratory that *Rm. vannielii* is capable of growing well on agar plates if incubated at 30°C in the dark in an atmosphere of 2% (v/v) oxygen.

This was achieved by inverting inoculated PMY plates in a 7.5 l sandwich box, and flushing the box with oxygen-free nitrogen for 30 minutes through a syringe needle inserted into the lid. 400 ml of air was then immediately injected into the box, and the inlet and outlet holes were sealed with insulating tape. Insulating tape was also passed round the join between box and lid to ensure that there was no diffusion of gases into or out of the box. Finally, the box was covered with aluminium foil and incubated at 30°C for 8-14 days.

*Caulobacter crescentus* was grown in a 50 ml culture volume in 250 ml conical flasks, shaking aerobically at 30°C. Growth was monitored by measuring the optical density of the culture at 550 nm.

*Staphylococcus aureus* and *Escherichia coli* were routinely incubated aerobically at 37°C in a shaking incubator. Growth of *E. coli* was measured by recording the optical density of the culture at 600 nm.

## 2.5 Maintenance of Cultures

*Rm. vannielii* and other members of the *Rhodospirillaceae* were maintained

as stab cultures in PHV agar deeps contained in universals. After inoculation from exponential growth phase cultures they were incubated at 30°C for 3-7 days under an incident light intensity of  $35 \mu\text{Em}^{-2}\text{s}^{-1}$ , then kept at room temperature.

Exponential phase cultures of *Caulobacter crescentus* were stored at 20°C in 15% (v/v) sterile glycerol.

*E. coli* strains were maintained as stab cultures in half strength LB medium, 0.7% (w/v) agar deeps contained in 2 ml glass screw cap tubes. After inoculation, they were incubated at 37°C overnight with the caps slightly loose. The following day, the caps were tightened, the tubes were sealed round the lid with Nascofilm and stored at room temperature in the dark.

In addition to other more short term methods, all strains were stored as frozen exponential phase cultures in 15% (v/v) sterile glycerol at 20°C.

Culture purity was checked by phase contrast microscopy and streaking to obtain single colonies on agar plates.

## 2.6 Preparation of Synchronised Swarmer Cell Populations

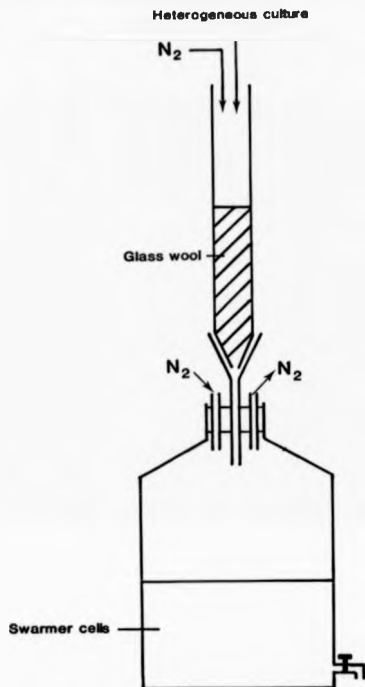
Swarmer cell populations of *Rm. vannielii* were prepared by passing a late exponential phase culture through a glass wool column using a modified version of the method described by Whittenbury and Dow (1977).

An 80 cm long x 7 cm diameter glass column was packed to a depth of 33 cm with glass wool (BDH), covered in aluminium foil and sterilized with dry heat at 160°C for a minimum of 2 hours. The column was washed with 1 l of sterile distilled water, and flushed continuously with oxygen-free nitrogen. It was then positioned above a glass collecting pot with sample port, which was also covered in aluminium foil and flushed continuously with oxygen-free nitrogen (see Fig. 2.1).

A 5 l culture of *Rm. vannielii* with an optical density of 540 nm (O.D.<sub>540</sub>) of between 1.0 and 2.0 was passed down the column. Swarmer cells passed through the glass wool, whereas multicellular arrays and stalked cells remained in the column. The O.D.<sub>540</sub> of the swarmer cell population was usually between 0.15 and 0.25.

Immediately the liquid had passed through the column, a bung with 2 metal tubes passing through it was pushed into the neck of the collecting vessel, and the headspace was gassed with oxygen-free nitrogen for 15 minutes. Then 2 football bladders filled with about 2 l of oxygen free nitrogen were rapidly attached to the metal tubes; their purpose being to allow removal of samples without disturbing the anaerobic atmosphere above the swarmer cell population. A small sample was taken, and the degree of homogeneity of the population assessed by light microscopy and cell volume distribution analysis (section 2.7).

The culture was incubated anaerobically with stirring at 30°C. When the aluminium foil was removed, the population was exposed to an incident light intensity of  $35 \mu\text{Ein}^{-2}\text{s}^{-1}$ , which triggered synchronous differentiation. The degree of synchrony of the population was assessed throughout an experiment by light microscopy and cell volume



**Fig. 2.1** Synchronisation of swarmer cells using a glass wool column  
(modified from Whittenbury and Dow, 1977).

distribution analysis.

## 2.7 Cell Volume Distribution Analysis

A Coulter counter model ZBI and Coulter channelyzer C1000 connected to a BBC microcomputer were used to determine cell size distributions.

100  $\mu$ l of sample was diluted in 20 ml of balanced salt electrolyte (Isoton, Coulter Electronics Ltd.) and profiles obtained using a 30  $\mu$ m orifice probe and an amplification setting of 0.5. The profiles were printed on a Tandy TRS-80 plotter. Latex particles of known size were used as calibration standards for cell volume analysis.

## 2.8 Light Microscopy

Light microscopy was performed using an Olympus microscope set up for phase contrast.

Photomicrographs were taken using a Leitz Dialux 22/22 EB microscope fitted with a Leitz Vario Orthomat 2 automatic microscope camera, on Kodak Panatomic X film. They were developed in Kodak D19 developer and fixed with Unifix.

## 2.9 Electron Microscopy

Samples were examined in a Jeol JEM-100S transmission electron

microscope at an accelerating voltage of 80 kV. They were supported on Formvar coated copper grids (300 square mesh, 3.05 mm diameter, Agar Aids) and negatively stained with 1% (w/v) phosphotungstic acid, brought to pH 7.0 with potassium hydroxide. Formvar and phosphotungstic acid were from Agar Aids. Photographs were taken using Kodak 4489 Ester thick base electron microscope film, which was developed in Kodak D19 developer and fixed in Unifix.

#### 2.10 Pulse Labelling Cell Proteins with L-[<sup>35</sup>S] methionine

L-[<sup>35</sup>S] methionine was obtained at a specific activity of >800 Ci mmol<sup>-1</sup> (Amersham International p.l.c.) and diluted with distilled water such that 100  $\mu$ Ci were contained in 1 ml. While cells were incubating anaerobically in the light with magnetic stirring at 30°C, the radiolabel was injected through the Suba seal to give an activity of 1  $\mu$ Ci ml<sup>-1</sup>. Cell suspensions were then further incubated for 20 minutes routinely, after which time unlabelled L-methionine was injected to a final concentration of 1 mM to stop further incorporation of radiolabel (Porter, 1984). Cells were then harvested by centrifugation, resuspended in the desired volume of 10 mM Tris HCl pH 7, and if necessary, stored at -20°C.

#### 2.11 Preparation of Cell Free Extracts

Cells were suspended in a small volume of 10 mM Tris HCl pH 7. The suspension was subjected to five 20 second bursts of sonication, using a 150 W MSE ultrasonic disintegrator at an amplitude of 6 microns, with a

3 mm diameter probe. One minute's cooling in an ice bath was allowed between bursts. After centrifugation at 10,000 g in an Eppendorf microcentrifuge for 15 minutes, the supernatant was removed and retained. Cell free extracts were stored at  $-20^{\circ}\text{C}$  if necessary.

#### 2.12 Measurement of Radioactivity of Cell Free Extracts

2  $\mu\text{l}$  samples of cell free extract were placed in mini scintillation vials. 3 ml of Beckman EF scintillation cocktail was added, and samples were counted in an LKB Minibeta scintillation counter.

#### 2.13 Determination of Protein Concentration

Protein assays were carried out using the Folin phenol reagent (Lowry et al., 1951) with crystalline bovine serum albumin as standard.

The following solutions were prepared:

- A. 5% (w/v) sodium carbonate ( $\text{Na}_2\text{CO}_3$ )
- B. 1% (w/v) copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ )
- C. 2% (w/v) sodium potassium tartrate [ $\text{NaK}(\text{CHOHCOO})_2$ ]
- D. To 50 ml of A was added 1 ml of B and 1 ml of C.
- E. Folin Ciocalteu reagent (Fisons) was diluted 1:1 with distilled water.
- F. 1 M sodium hydroxide ( $\text{NaOH}$ ).

Samples were diluted in 0.5 ml of water in chromic acid-washed test



tubes. 0.5 ml of sodium hydroxide was added, and samples were boiled for 5 minutes and cooled. 2.5 ml of solution D was added to each sample and they were incubated for 10 minutes at room temperature. Subsequently 0.5 ml of Folin Ciocalteu reagent was added with thorough vortexing and samples were allowed to stand at room temperature for 30 minutes. The absorbance of the samples at 750 nm was then measured against a reagent blank containing 0.5 ml of distilled water, and their protein content determined by reference to a calibration graph of absorbance against bovine serum albumin concentration.

#### 2.14 Preparation of Flagella

Flagella were prepared in the following two ways from a 5 l late exponential phase culture of *Rm. vannielii* grown anaerobically in the light.

(1) Cells were removed by centrifugation at 17,400 g for 30 minutes at 4°C. The supernatant was retained, and kept at 4°C. It was then concentrated approximately 100 times using an Amicon model CH4 hollow fibre concentrator fitted with a hollow fibre cartridge with a molecular weight cut-off of 100,000. This concentrate (around 50 ml) was adjusted so that it was 35% (w/v) CsCl, 0.015 M Tris HCl pH 7.8 and 2% (v/v) Triton X-100. This was dispensed into 25 ml polycarbonate threaded centrifuge tubes and centrifuged at 101,200 g in the 8 x 25 ml titanium rotor of an MSE 65 ultracentrifuge for 50 hours at 4°C. Flagella formed a clearly visible white band which was collected by fractionating the gradient from the bottom, and dialysed overnight against 10 mM Tris HCl pH 7.8, 10 mM EDTA.

(11) The culture supernatant was obtained in the same way. It was then stirred at 4°C, and solid  $(\text{NH}_4)_2\text{SO}_4$  was added to 30% saturation (16.4 g/100 ml). After stirring at 4°C for 2 hours, the precipitate was collected by centrifugation at 25,000 g for 30 minutes at 4°C. It was then resuspended in a small volume of 10 mM Tris HCl pH 7.8, 10 mM EDTA and dialysed overnight against the same buffer.

These methods were adapted from Lagenaur and Agabian (1976).

#### 2.15 Preparation of Hooks

Hooks were prepared from a preparation of shed flagella by acid dissociating the flagellar filament and collecting hooks by centrifugation (Lagenaur *et al.*, 1978).

The pH of the flagella suspension was lowered to 3.5 by addition of glacial acetic acid at room temperature. It was then centrifuged at 30,000 g for 10 minutes at 4°C, and the pellet was resuspended in 1 ml of buffer A (10 mM Tris HCl pH 8, 10 mM EDTA, 0.5% (v/v) Triton X-100). Two linear 15-30% (w/v) sucrose gradients in buffer A were poured in Beckman polyallomer 5.5 ml centrifuge tubes, using a small MSE gradient maker and a peristaltic pump. 0.5 ml of sample was layered on to each gradient, and they were centrifuged in the SW50.1 rotor of a Beckman L8 ultracentrifuge at 90,000 g for 3 hours at 4°C. The gradients were fractionated by piercing the bottom of the tube and collecting 0.33 ml fractions.

## 2.16 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Two types of fully denaturing SDS polyacrylamide gels were used during this study. These were a uniform concentration 10% (w/v) polyacrylamide slab gel and a 10-30% (w/v) polyacrylamide exponential gradient slab gel. In general, the gradient gels were used for looking at a complex mixture of proteins with a large range of molecular weights, while 10% (w/v) polyacrylamide gels were employed when the molecular weights of the proteins of interest fall between approximately 15 k and 70 k (Hames and Rickwood, 1981).

Stock solutions were prepared as follows:

### Resolving gel buffer pH 8.8

Tris 36.33 g (Sigma)

Conc. HCl to bring pH to 8.8

Double distilled water to 100 ml

### Stacking gel buffer pH 6.8

Tris 5.98 g

Conc. HCl to bring pH to 6.8

Double distilled water to 100 ml

### 60% (w/v) acrylamide (high bis)

Acrylamide 60 g (Eastman Kodak)

Bisacrylamide 1.6 g (Biorad)

Double distilled water to 100 ml

Acrylamide	60 g
Bisacrylamide	0.3 g
Double distilled water to	100 ml

Acrylamide	10 g
Bisacrylamide	0.5 g
Double distilled water to	100 ml

Tris	30.2 g
Glycine	144 g (Biorad)
Double distilled water to 1000 ml	

The reservoir buffer used consisted of:

5x stock	300 ml
10% (w/v) sodium dodecyl sulphate (SDS)	15 ml
Double distilled water to 1500 ml	

The gels were cast between clean glass plates measuring 20 cm x 25 cm, holding a resolving gel volume of 75 ml. A stacking gel of 3% (w/v) polyacrylamide was cast on top of the resolving gel.

The gel mixtures were as follows:

10% (w/v) acrylamide mixture - 75 ml

High bis acrylamide stock	12.5 ml
Double distilled water	52.3
Resolving gel buffer stock	9.4
10% (w/v) SDS	0.75

This was mixed, degassed under vacuum in a desiccator and immediately before pouring 15  $\mu$ l of TEMED (N,N,N,N'-tetramethylethylenediamine) (Eastman Kodak or Biorad) and 150  $\mu$ l of 10% (w/v) ammonium persulphate (BDH) added.

30% (w/v) acrylamide mixture - 20 ml

Low bis acrylamide stock	10 ml
75% (v/v) glycerol	7.3
Resolving gel buffer stock	2.5
10% (w/v) SDS	0.2

This was mixed, degassed under vacuum in a desiccator, and immediately before pouring 4  $\mu$ l of TEMED and 40  $\mu$ l of 10% (w/v) ammonium persulphate added.

10% (w/v) acrylamide mixture - 50 ml for gradient gels

High bis acrylamide stock	8.3 ml
Double distilled water	34.9
Resolving gel buffer stock	6.25
10% (w/v) SDS	0.5

This was mixed and degassed in the same way and immediately before pouring 10  $\mu$ l of TEMED and 100  $\mu$ l of 10% (w/v) ammonium persulphate was

added.

Stacking gel mixture - 10 ml

Stacking gel acrylamide stock	3.0 ml
Double distilled water	4.4
Stacking gel buffer	2.4
10% (w/v) SDS	0.1

This was mixed and degassed, and immediately before pouring 5  $\mu$ l of TEMED and 100  $\mu$ l of 10% (w/v) ammonium persulphate was added.

For a 10% (w/v) polyacrylamide gel, 75 ml of acrylamide mixture was poured between glass plates separated by PTFE spacers, sealed by polypropylene tubing smeared with paraffin wax, and held together by bulldog clips.

For a 10-30% (w/v) polyacrylamide gradient gel, 20 ml of 30% (w/v) acrylamide mixture was placed in a 20 ml mixing chamber, and this was stoppered with a Suba seal. The 10% (w/v) acrylamide mixture was pumped into the 30% (w/v) acrylamide mixture using a peristaltic pump, and thoroughly mixed by a magnetic stirrer in the mixing chamber. Glycerol was included in the 30% (w/v) acrylamide mixture to produce a density gradient which minimised mixing due to convection caused by the heat evolved during polymerisation. During pouring the volume of liquid in the mixing chamber remained constant and was continuously diluted by 10% (w/v) gel solution. The gradient of acrylamide was pumped between the glass plates assembled as previously described.

After pouring, the gel solution was overlaid with water saturated

butanol and allowed to set. The butanol was then washed off and a stacking gel set above the resolving gel with a PTFE slot-former pushed into it. When the stacking gel had set, the slot-former and polypropylene tubing were removed and the gel placed in the tank.

#### Sample preparation

Samples were made 0.0625 M with respect to Tris HCl pH 6.8, 10% (w/v) with respect to SDS, 0.78 M with respect to  $\beta$ -mercaptoethanol and 0.005% (w/v) with respect to bromophenol blue. This mixture, in a volume of between 5 and 100  $\mu$ l, was boiled for 3 minutes, cooled, then loaded into the gel slots using a Hamilton syringe.

#### Electrophoresis conditions

Electrophoresis was carried out in a vertical perspex Studier type gel tank at 4°C. Air bubbles were removed from beneath the gel using a syringe fitted with a bent hypodermic needle. Electrophoresis was overnight at 8 mA for 10% (w/v) polyacrylamide gels and 20 mA for 10-30% (w/v) gradient gels.

When the bromophenol blue tracker dye reached the bottom of the gel, the gel was removed from the plates and fixed as required.

#### Standards

Protein standards of known molecular weight were included on gels, and a linear calibration graph was obtained by plotting  $\log_{10}$  molecular weight against  $R_F$ . In this way, the approximate molecular weight of sample proteins was determined.

## 2.17 Staining Proteins in Polyacrylamide Gels

### 2.17.1 Coomassie blue staining

The staining solution was 45% (v/v) methanol, 10% (v/v) glacial acetic acid and 1% (w/v) Coomassie brilliant blue R250 (Biorad). Fixing and staining occur simultaneously. Gels were immersed in stain with agitation for around 4 hours, and destained in 45% (v/v) methanol, 10% (v/v) glacial acetic acid until the background was almost colourless. Gels were stored in 10% (v/v) isopropanol, 10% (v/v) glacial acetic acid.

### 2.17.2 Silver staining (Wray *et al.*, 1981)

Silver staining is considerably more sensitive than Coomassie blue staining, and the following method was used routinely. It is important to use water of the highest purity available, and to this end double distilled water was used throughout the procedure.

Gels were immersed in 50% (v/v) methanol, with agitation, for at least 8 hours, during which period the 50% (v/v) methanol solution was changed 4-5 times. This fixed the proteins in the gel, and also washed out substances which interfered with the silver stain, possibly glycine and glycerol.

To make the staining solution, the following solutions were prepared:

- A. 1.6 g silver nitrate (Johnson Matthey Chemicals Ltd.) dissolved in 8 ml of water.
- B. 42 ml of 0.36% (w/v) sodium hydroxide mixed with 2.5 ml of "0.88" ammonia solution (BDH).



The staining solution was then prepared by slowly adding A to B with constant swirling. The mixture was then made to 200 ml with water.

The gel was immersed in staining solution, with agitation, for 15 minutes, then washed in 2 changes of water for 5 minutes each. Protein bands were visualised by soaking the gel in a developer solution, consisting of 2.5 ml of 1% (w/v) citric acid and 0.6 ml of 37% (v/v) formaldehyde solution (BDH) made up to 500 ml with water. After 10-15 minutes protein bands appeared on a colourless background. When the bands reached the desired intensity, the gel was transferred to, and stored in, a stop solution consisting of 45% (v/v) methanol, 10% (v/v) glacial acetic acid.

#### 2.18 Photography of Polyacrylamide Gels

Polyacrylamide gels were placed on a light box and photographed with a Pentax K-1000 camera loaded with Kodak Panatomic X film. Film was developed in Kodak D19 developer for 3 minutes and fixed in Unifix. Prints were made on Kodabrome paper.

#### 2.19 Autoradiography

Gels containing  $^{35}\text{S}$  or  $^3\text{H}$  were dried on to Whatman 3 MM filter paper, under vacuum at 80°C for gels without a fluor, and 60°C for gels with a fluor (section 2.20), in a Biorad gel drier. They were then exposed to Kodak X-Omat S X-ray film in a Harmer film cassette.

Nitrocellulose filters carrying  $^{32}\text{P}$  or  $^{125}\text{I}$  were air-dried, supported on cardboard and covered in cling film. They were then exposed to Fuji RX X-ray film in a film cassette with a Dupont Cronex Lightning Plus intensifying screen placed on top of the film. Cassettes were held at  $-70^{\circ}\text{C}$  during the exposure.

Films were developed in Kodak LX24 X-ray developer for 3 minutes and fixed in Kodak FX40 X-ray fixer.

## 2.20 Fluorography

Fluorography, or impregnation of gels with a fluor, allows the detection of  $^3\text{H}$  labelled proteins when exposed to X-ray film, and increases the sensitivity of X-ray film to  $^{35}\text{S}$  labelled proteins.

In this study, it was carried out according to the method of Skinner and Griswold (1983). After electrophoresis, polyacrylamide gels were fixed in glacial acetic acid for 5 minutes, then soaked in a 20% (w/v) solution of 2,5 diphenyloxazole (PPO, from BDH) in glacial acetic acid for 1.5 hours, with gentle agitation. Gels were then transferred into distilled water for 30 minutes, with agitation, during which time the PPO precipitated in the gel. Gels were then ready for drying.

## 2.21 Protein Purification by Electrophoresis

The purification of flagellin for antibody production was undertaken by preparative gel electrophoresis (Porter, 1984).

The wells of a 10% (w/v) polyacrylamide SDS gel were all loaded with flagella solubilised in SDS sample buffer (62.5 mM Tris HCl pH 6.8, 10% (w/v) SDS, 0.78 M  $\beta$ -mercaptoethanol and 0.005% (w/v) bromophenol blue), with the exception of two wells, which were loaded with protein molecular weight standards. After electrophoresis, the tracks containing the standards and one containing solubilised flagella were separated from the rest of the gel using a scalpel. This gel strip was silver stained, while the remainder of the gel was stored at  $-20^{\circ}\text{C}$ . The position of flagellin in the unstained gel was calculated from the distance migrated by the stained flagellin, taking into account the relative lengths of the stained and unstained gel portions. The flagellin band was then cut out with a scalpel, and protein was removed from the gel strip by upward electrophoresis through a stacking gel (Mandel-Hartvig, 1982).

This method used the same buffers and equipment described for SDS PAGE with a few modifications. When the gel plates were sandwiched together, a double thickness of PTFE spacer was used, to allow the excised gel slices to move freely between the plates. A supporting gel of 10% (w/v) polyacrylamide in running buffer was cast, and the gel strip to be eluted was placed on this after being incubated briefly with 0.5% (w/v) bromophenol blue. Then a stacking gel of about 2 cm was cast on top of the excised gel strip, and 2 plugs of 1% (w/v) agarose in running buffer were used to seal any gap between the polyacrylamide and the spacers (see Fig. 2.2). This prevented sample escaping down the sides of the gel.

Electrophoresis was carried out at 20 mA and  $4^{\circ}\text{C}$  with the anode connected to the top of the gel. In this way, the protein was

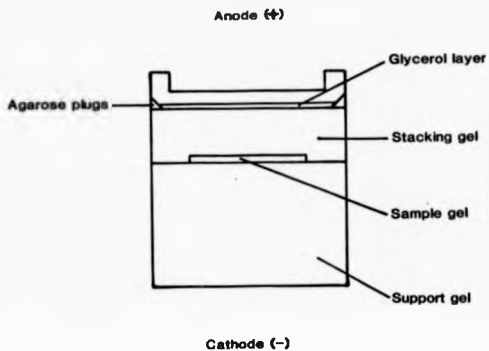


Fig. 2.2 Elution of protein from a polyacrylamide gel by upward electrophoresis through a stacking gel (from Mendel-Hartvig, 1982).

electrophoresed through the stacking gel and collected in an overlay of 2 ml of 30% (w/v) glycerol in stacking gel buffer, visualised by the bromophenol blue. The sample was subsequently collected with a syringe.

## 2.22 Preparation of Antisera

Antisera were raised in New Zealand white rabbits. A blood sample was taken from the ear before injection; this contained pre-immune serum.

Between 10 and 200  $\mu$ g of protein in 1 ml of phosphate buffered saline (PBS) was mixed with 1 ml of Freund's complete adjuvant by passing the mixture repeatedly between two glass syringes. The resulting stiff white emulsion was injected subcutaneously into the back of the neck. 21 days later, a booster injection of the same protein sample mixed with 1 ml of Freund's incomplete adjuvant was administered, and this was repeated after 7 days. One week later, the rabbits were bled. Blood was taken twice a week for several weeks, and pre-immune and post-immune sera were stored in aliquots at  $-20^{\circ}\text{C}$ .

## 2.23 Rocket Immunelectrophoresis

Rocket immunelectrophoresis was carried out in 1% (v/v) agarose gels cast on microscope slides. The agarose was dissolved in a buffer of 50 mM sodium barbital, 10 mM barbituric acid pH 8.6 (Sigma) and Triton X-100 was added to a final concentration of 1% (v/v) when the molten agarose had cooled to  $55^{\circ}\text{C}$ . Just before pouring, antiserum was added to a concentration of between 0.5% (v/v) and 2% (v/v), and mixed.

The reservoir buffer was 50 mM sodium barbital, 10 mM barbituric acid pH 8.6 containing 1% (v/v) Triton X-100, and the gels were run in a Shandon 600 horizontal electrophoresis chamber, with Whatman 3 MM filter paper wicks connecting gel and reservoir buffer. Between 2 and 12  $\mu$ g of antigen were made 1% (v/v) with respect to Triton X-100 and loaded into circular wells cut in the agarose in a volume of up to 20  $\mu$ l. Electrophoresis towards the anode was carried out at 70 V overnight at room temperature.

After electrophoresis the gels were blotted dry for 10 minutes, washed twice in PBS for 15 minutes to remove uncomplexed proteins, and blotted dry. Finally they were washed in distilled water for 15 minutes to remove salt, blotted, and completely dried on to the microscope slides in an oven. The slides were then stained in 1% (w/v) Coomassie brilliant blue R250 in 45% (v/v) methanol, 10% (v/v) glacial acetic acid for 5 minutes, and destained in 45% (v/v) methanol, 10% (v/v) glacial acetic acid.

#### 2.24 Radioiodination of Protein A

When the following procedure is carried out,  $\text{Na}^{125}\text{I}$  is oxidised by the catalyst chloramine T in the presence of Protein A, with the subsequent incorporation of  $^{125}\text{I}$  into the tyrosine residues of the protein in high yield (Bolton, 1977).

25  $\mu$ l of a 1 mg  $\text{ml}^{-1}$  solution of Protein A, 1 mCi  $^{125}\text{I}$  as free iodide (250-600 mCi  $\text{ml}^{-1}$ , Amersham International p.l.c.) and 10  $\mu$ l of a freshly prepared 2 mg  $\text{ml}^{-1}$  solution of chloramine T in 0.5 M phosphate buffer pH

7.5 were mixed and incubated at room temperature for 2 minutes. The following solutions were then added: 25  $\mu$ l of a 2 mg ml<sup>-1</sup> solution of tyrosine in phosphate buffer, 50  $\mu$ l of 10% (w/v) bovine serum albumin (Pentax Fraction V, Sigma) and 200  $\mu$ l of PBS. This was then applied to a 5 ml column of Sephadex G50 medium grade (Pharmacia) of bed height 20 cm, equilibrated in PBS and poured in a 5 ml pipette. The purpose of the column was to separate radioiodinated Protein A from free iodine, and the protein was contained in the first peak of gamma radiation to come off the column. The column had been prewashed with 10 ml of 1% (w/v) BSA in PBS to minimise the adsorption of the very low concentrations of labelled protein to be separated. When the sample had run into the column, it was eluted with PBS, and approximately 5 ml of eluate was collected in 0.5 ml fractions. The fractions were monitored with a gamma radiation monitor and one or two containing iodinated Protein A were stored at 4°C.

The composition of the phosphate-buffered saline (PBS) used was: 138 mM NaCl, 2.8 mM KCl, 7.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. The 0.5 M phosphate buffer pH 7.5 was made by preparing 0.5 M KH<sub>2</sub>PO<sub>4</sub> and 0.5 M K<sub>2</sub>HPO<sub>4</sub>, and adding KH<sub>2</sub>PO<sub>4</sub> to K<sub>2</sub>HPO<sub>4</sub> to bring the pH to 7.5.

## 2.25 Western Blotting

### 2.25.1 Transfer of proteins separated in a polyacrylamide gel to nitrocellulose

A nitrocellulose membrane filter (Schleicher and Schuell, BA85) was cut to the size of the polyacrylamide gel, soaked in Western transfer buffer (25 mM Tris HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol) and placed

on top of the gel. Care was taken to remove air bubbles between gel and nitrocellulose. Using a Biorad Transblot tank and power supply according to the manufacturer's instructions, transfer of proteins from gel to nitrocellulose was carried out at 300 mA for 2.5 hours.

After transfer, the nitrocellulose filter was soaked in 3% (w/v) bovine serum albumin (Sigma) in PBS in a sandwich box, and shaken on a platform shaker for 1 hour, to allow protein to bind non-specifically to the nitrocellulose. Antiserum at a dilution of 1:1000 was added and the filter was allowed to shake in this solution for between 1 hour and overnight, at room temperature. The filter was then washed 3-4 times in PBS.

#### 2.25.2 Detection of antigen-antibody complexes with radioiodinated Protein A

A fresh solution of 3% (w/v) BSA in PBS was poured into the sandwich box containing the filter, and to this 10  $\mu$ l of radiolodinated Protein A containing more than  $10^6$  counts per minute was added. The filter was shaken in this solution for 2 hours at room temperature, and then washed several times in 1% (v/v) Triton X-100 in PBS until the background count measured with the gamma radiation monitor was low. The filter was then exposed to X-ray film, with an intensifying screen.

#### 2.25.3 Detection of antigen-antibody complexes using a horseradish peroxidase colour reaction

A fresh solution of 1% (w/v) BSA in PBS was added to the filter, and biotinylated Protein A (Amersham International p.l.c.) was added at a dilution of 1:2000. The filter was shaken in this solution for 1 hour at room temperature, followed by five 5 minute washes in 1% (v/v) Triton



X-100 in PBS. Then the 1% (w/v) BSA in PBS was replaced and streptavidin-biotinylated horseradish peroxidase complex (Amersham International p.l.c.) added at a dilution of 1:400. This was shaken for 20 minutes at room temperature, after which the filter was washed three times for 5 minutes in 1% (v/v) Triton X-100 in PBS. Finally a solution of  $0.5 \text{ mg ml}^{-1}$  diaminobenzidine (Sigma) and 0.03% (w/v) nickel chloride (Sigma) in PBS containing 0.02% (v/v) hydrogen peroxide (Fisons) was added to the box containing the filter. This was shaken until the desired intensity of blue colour developed, and the reaction was stopped by transfer of the filter to 1% (v/v) Triton X-100 in PBS.

## 2.26 Immunoprecipitation

Formalin treated, heat killed *Staphylococcus aureus* Cowan serotype 1 cells were prepared according to Kessler (1975), and stored at  $-20^{\circ}\text{C}$ .

Detergent buffer A was made up at double strength and diluted when required. Its constituents were as follows:

100 mM	KCl
5 ml	$\text{MgCl}_2$
100 mM	Tris HCl pH 8.2
1% (w/v)	Triton X-100
1% (w/v)	sodium deoxycholate
0.5% (w/v)	SDS

Buffer B was:

100 mM	KCl
5 mM	MgCl <sub>2</sub>
100 mM	Tris HCl pH 8.2
1% (w/v)	Triton X-100

50  $\mu$ l of antiserum, 50  $\mu$ l of [<sup>35</sup>S] methionine labelled cell free extract containing around 10<sup>6</sup> counts per minute and 950  $\mu$ l of 2x buffer A were combined in a 1.5 ml Eppendorf microcentrifuge tube, mixed and incubated for 1 hour at 30°C.

During this time, the required number of aliquots of formalin treated *S. aureus* cells were thawed. Each 1 ml aliquot of cells was washed four times in 300  $\mu$ l of buffer B and finally resuspended in 360  $\mu$ l of buffer B. 100  $\mu$ l of this was added to each immunoprecipitation, and they were incubated overnight at 4°C with agitation.

The following day the suspensions were centrifuged in an Eppendorf microfuge for 2 minutes, and the supernatants discarded. The immunoprecipitates were then washed six times in 250  $\mu$ l of buffer A. After the final wash, the pellet was resuspended in 30  $\mu$ l of SDS sample buffer (62.5 mM Tris HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.7% M  $\beta$ -mercaptoethanol and 0.005% (w/v) bromophenol blue), and boiled for 5 minutes. The effect of this was to release labelled protein bound to antibody carried on the killed *S. aureus* cells into solution in the sample buffer, and whole cells and cell debris were removed by centrifugation at 10,000 g for 4 minutes. The supernatant was carefully removed, and stored at -20°C prior to polyacrylamide gel electrophoresis.

## 2.27 Assay for Methyl-Accepting Chemotaxis Proteins

This *in vivo* assay for protein methylation in the absence of protein synthesis was described by Shaw *et al.*, 1983.

Cultures grown in minimal medium were treated with chloramphenicol ( $50 \mu\text{g ml}^{-1}$ ) for 5 minutes, and then incubated for 20 minutes at  $30^\circ\text{C}$  in the presence of either  $15 \mu\text{Ci ml}^{-1}$  or  $75 \mu\text{Ci ml}^{-1}$  of L-[methyl  $^3\text{H}$ ] methionine ( $1 \text{ mCi ml}^{-1}$ , Amersham International p.l.c.). Samples were then immediately mixed with an equal volume of ice cold acetone, and centrifuged at  $34,000 \text{ g}$  for 30 minutes at  $4^\circ\text{C}$  in an MSE H1 Spin 21 centrifuge. The resulting precipitates were washed with 50% (v/v) ice cold acetone and air dried. They were then resuspended in a small volume of  $10 \text{ mM}$  Tris HCl pH 7.5 and their protein content was measured, prior to separation in 10% (w/v) polyacrylamide SDS gels.

## 2.28 DNA Isolation

### 2.28.1 Preparation of chromosomal DNA from members of the Rhodospirillaceae and *Caulobacter crescentus*

This method was used to prepare DNA from *Rm. vannielii* and several other purple non-sulphur bacteria. The acetone treatment was excluded when *C. crescentus* DNA was prepared. The initial part of the procedure brought about cell lysis, then DNA was purified and concentrated by caesium chloride density gradient centrifugation.

TES buffer was  $10 \text{ mM}$  Tris HCl pH 8,  $1 \text{ mM}$  EDTA,  $100 \text{ mM}$  NaCl and TE buffer was  $10 \text{ mM}$  Tris HCl pH 8,  $1 \text{ mM}$  EDTA. Approximately  $1 \text{ g}$  (wet weight) of

cells was resuspended in 5 ml TES buffer. Half a volume of acetone was added, the suspension was thoroughly vortexed, and incubated at room temperature for 10 minutes. Cells were then pelleted at 4500 g in an MSE Multax centrifuge, and washed 3 times in TES buffer. The final pellet was resuspended in 10 ml TES buffer, and solid lysozyme (Sigma) was added to a final concentration of  $1 \text{ mg ml}^{-1}$ . This was mixed gently by inversion and incubated at  $37^{\circ}\text{C}$  for 1 hour. Then sarkosyl (BDH) was added to a final concentration of 6% (v/v), and the suspension was vortexed and incubated at  $60^{\circ}\text{C}$  for 15 minutes. At this stage the turbid suspension had become a transparent lysate; cell lysis was complete as judged visually and by light microscopy.

19 g of caesium chloride (Fisons) was weighed into an MSE 25 ml polycarbonate threaded centrifuge tube. Lysate (around 12 ml) was added until the contents of the centrifuge tube weighed 34 g. The metal cap was firmly screwed on, and the tube was inverted until the CsCl dissolved. Centrifugation was at 181,000 g for 36 hours at  $15^{\circ}\text{C}$  in an 8 x 25 ml titanium rotor of an MSE 65 ultracentrifuge. The centrifuge was allowed to slow down with the brake off, and the gradients were fractionated by piercing the bottom of the tube and allowing the contents to drip out slowly. Approximately half way up the tube, the fluid would become extremely viscous. It would cease to drip out, and emerged from the bottom of the tube as a continuous, sticky gel. This viscous material was retained and dialysed extensively against TE buffer.

#### 2.28.2 Large scale plasmid preparation

This was done using the method of Clewell and Helinski, 1970.

Growth of *Escherichia coli* harbouring the desired plasmid and amplification of the plasmid using a high concentration of antibiotic was as in Molecular Cloning: A Laboratory Manual (Maniatis *et al.*, 1982), with the exception that spectinomycin was added to a final concentration of  $300 \mu\text{g ml}^{-1}$  instead of chloramphenicol to  $170 \mu\text{g ml}^{-1}$ .

To obtain good separation and yield of plasmid DNA, it is necessary to lyse the cells gently in sucrose, such that plasmid DNA will leak out of the cell, but most of the chromosomal DNA remains attached to the cell membrane. Cells were pelleted at 17,400 g for 10 minutes at room temperature and resuspended in 16.5 ml of 0.05 M Tris HCl pH 8, 25% (w/v) sucrose. 25  $\mu\text{g}$  of lysozyme was gently dissolved in this, and the suspension was placed on ice for 5 minutes, with occasional gentle mixing. 18 ml of a lysis mix containing 0.05 M Tris HCl pH 8, 62.5 mM EDTA, 2% (w/v) Brij 58 (Sigma) and 0.4% (w/v) sodium deoxycholate was then added. The mixture was alternately inverted gently and incubated at  $42^{\circ}\text{C}$  for 15 minutes, until cell lysis had occurred. The lysate was then centrifuged at 43,000 g for 15 minutes at  $4^{\circ}\text{C}$  in sterile polypropylene Oak Ridge tubes. A large fluffy pellet of gently lysed, osmotically stabilised cells and chromosomal DNA formed, and the top 15 ml of supernatant was removed with a pipette and retained.

Separation of the plasmid DNA and the chromosomal DNA in this cleared lysate was achieved by caesium chloride density gradient centrifugation in the VT1 65 rotor of a Beckman L8 ultracentrifuge. For each 5.1 ml ultracentrifuge tube 4.75 g of CsCl was dissolved in 5 ml of cleared lysate and 0.17 ml of a 2.5% (w/v) solution of ethidium bromide (Sigma) added. This solution was dispensed into Beckman Quick Seal Ultra Clear tubes, which were heat sealed and centrifuged at 310,000 g overnight at

15°C. The following morning the tubes were illuminated with a long wavelength U.V. hand-held wand in the dark room, to visualize the DNA complexed with ethidium bromide. The lower of the 2 bands contained plasmid DNA and was removed using a 1 ml disposable syringe and needle. This was extracted three times with water saturated butanol to remove the ethidium bromide, and dialysed extensively against TE buffer.

#### 2.28.3 Rapid small scale plasmid preparation

This is the alkaline lysis method. *E. coli* cells harbouring the plasmid were grown overnight in a universal containing 5 ml of LB medium and an appropriate antibiotic.

Cells were pelleted at 4,500 g for 10 minutes, resuspended in 1 ml 10 mM Tris HCl pH 7 and transferred to a 1.5 ml Eppendorf microcentrifuge tube. Cells were again pelleted and resuspended in 150 µl of 50 mM glucose, 10 mM EDTA, 25 mM Tris Cl pH 8. Lysozyme was added to a concentration of 4 mg ml<sup>-1</sup>, and incubation was for 5 minutes at room temperature. 200 µl of a freshly prepared solution of 0.2 M sodium hydroxide, 1% (w/v) SDS were then added, and after mixing 2-3 times by inversion and incubation on ice for 5 minutes, cell lysis was achieved.

150 µl of an ice cold solution of 5 M (with respect to acetate) potassium acetate was added. This was briefly and gently vortexed, and allowed to stand on ice for 5 minutes. After centrifugation at 10,000 g for 5 minutes, the supernatant was retained and extracted with phenol chloroform (section 2.30), followed by chloroform extraction. DNA was then ethanol precipitated and the pellet was dried in a desiccator and resuspended in 50 µl of TE buffer containing 20 µg ml<sup>-1</sup> DNase-free RNase.

## 2.29 Estimation of DNA Concentration and Purity

This was done spectrophotometrically in an LKB Ultrospec 4050, using 1 ml quartz cuvettes which had been washed thoroughly in distilled water and ethanol. 10  $\mu$ l of the DNA sample was mixed with 1 ml sterile distilled water in a cuvette, and the absorbance was read at 260 nm, 280 nm and 320 nm against a sterile distilled water blank.

The concentration of DNA in the sample could be estimated from the absorbance at 260 nm, assuming that an O.D.<sub>260</sub> of 1 is given by 50  $\mu$ g ml<sup>-1</sup> of double stranded DNA (Maniatis *et al.*, 1982). DNA was also examined on a 0.6% (w/v) agarose gel (section 2.35).

The absorbance at the other wavelengths were used to give an estimate of the purity of the DNA. If the DNA is free from contaminating RNA or protein the O.D.<sub>260</sub>:O.D.<sub>280</sub> ratio should be between 1.65 and 1.85. The absorbance at 320 nm of a nucleic acid solution should be zero; a reading of this wavelength is an indication of the light scattering due to particulate matter in the solution (Scheif and Wensink, 1981).

## 2.30 Preparation of Phenol Chloroform for Extraction of DNA

### Solutions

Phenol chloroform extraction and chloroform extraction of DNA were performed to remove unwanted protein (either contaminating cellular protein or spent enzyme) from DNA samples.

100 g of phenol crystals (BDH) were dissolved in 100 ml of chloroform.

4 ml of isoamyl alcohol and 0.1 g of 8-hydroxyquinoline were added. This solution was neutralised by extracting it thoroughly with 0.2 volumes of 1 M Tris HCl pH 8 twice, and 0.2 volumes of TE buffer twice. It was stored under 20 ml of TE buffer pH 8 at 4°C in a light proof bottle.

### 2.31 Ethanol Precipitation of DNA

DNA was ethanol precipitated when it was necessary to concentrate the DNA, remove traces of organic solvents or resuspend the DNA in a different buffer.

The DNA solution was made 0.3 M with respect to sodium acetate, using a stock solution of 3 M sodium acetate brought to pH 5.2 with glacial acetic acid. Then 2 volumes of ice cold ethanol were added, and the sample was vortexed and incubated at -20°C for between 2 hours and 16 hours. The sample was then centrifuged at 10,000 g for 15 minutes at 4°C, the supernatant carefully poured off, and the pellet dried under vacuum in a desiccator.

### 2.32 Restriction Enzyme Digestion of DNA

The system of dividing restriction enzymes into 3 groups: those requiring high salt buffers, those requiring medium salt buffers and those requiring low salt buffers was used (Maniatis *et al.*, 1982). The final concentration of the buffer constituents in the reaction were as follows:



<u>BUFFER</u>	<u>NaCl</u>	<u>Tris HCl pH 7.5</u>	<u>MgCl<sub>2</sub></u>	<u>Dithiothreitol</u>
Medium salt	50 mM	10 mM	10 mM	1 mM
High salt	100 mM	50 mM	10 mM	1 mM

These buffers were prepared as 10x concentrated stocks and stored at -20°C.

Restriction enzymes *EcoRI*, *Sall* and *HindIII* were used in this study, and reaction conditions were as in Table 2.4. Restriction enzymes were obtained from Amersham International p.l.c. or Boehringer Mannheim GmbH.

### 2.33 DNA Ligation

10x concentrated ligation buffer was prepared as follows:

0.66 M	Tris HCl pH 7.5
50 mM	MgCl <sub>2</sub>
50 mM	Dithiothreitol (DTT)
10 mM	Adenosine 5'-triphosphate from equine muscle (ATP, Sigma)

This was stored in aliquots at -20°C.

Ligation reactions contained the desired quantity of DNA, the appropriate volume of 10x ligation buffer and the required number of units of T<sub>4</sub> DNA ligase (Amersham International p.l.c.). They were incubated at 15°C for 2-6 hours.

Table 2.4   Reaction Conditions for Restriction Enzymes Used in This  
Study

ENZYME	BUFFER	INCUBATION TEMPERATURE	PERIOD OF INCUBATION
<i>EcoRI</i>	High salt	37°C	1-4 hours
<i>SaII</i>	High salt	37°C	1-4 hours
<i>HindIII</i>	Medium salt	37°C	1-4 hours

## 2.34 Transformation of *Escherichia coli* Cells With Plasmid DNA

Transformation of *E. coli* DH1 with plasmid DNA by the calcium chloride procedure was carried out according to Maniatis *et al.*, 1982.

## 2.35 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to size, and to assess the concentration of DNA samples and check for nuclease contamination.

0.6% (w/v) agarose (low electroendosmosis, Sigma) gels were cast in a mould consisting of an 11 cm x 12 cm perspex base plate with autoclave tape sides. A 50x concentrated stock of Tris acetate EDTA (TAE) buffer was prepared, containing per litre:

Tris	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA pH8	100.0 ml

0.6g of agarose was added to 100 ml of single strength TAE (0.04 M Tris acetate, 0.001 EDTA), and melted in a microwave oven. When this cooled to around 50°C, ethidium bromide was added to a final concentration of 0.5  $\mu\text{g ml}^{-1}$  (5  $\mu\text{l}$  of a 10  $\text{mg ml}^{-1}$  stock). The gel was then poured into the mould on a level surface, and a slot former was inserted approximately 1.5 cm from one end. 10x concentrated gel loading mix was 25% (w/v) Ficoll 400 (Sigma), 0.4% (w/v) bromophenol blue. The required amount of this was thoroughly mixed with each DNA sample in TE buffer.

The autoclave tape was removed from the gel, and the gel and baseplate were placed in a perspex horizontal electrophoresis tank filled with TAE buffer containing  $0.5 \mu\text{g ml}^{-1}$  ethidium bromide, such that the gel was just submerged. Samples were loaded into the wells using a  $20 \mu\text{l}$  Gilson pipette, and electrophoresis was carried out at 60V for several hours towards the anode, until the tracker dye reached the end of the gel.

The fragments generated from a HindIII digestion of bacteriophage  $\lambda$  DNA were used as size standards. The  $R_f$  was proportional to  $\log_{10}$  molecular weight.

#### 2.36 Photography of Agarose Gels

DNA ethidium bromide complexes in agarose gels were visualised by placing the gel on an ultra violet light transilluminator. Photographs were taken with a Polaroid camera, using Polaroid Type 665 film.

#### 2.37 Recovery of DNA from Agarose

DNA fragments separated by agarose gel electrophoresis were recovered from gels cast with low melting temperature agarose. This agarose can be dissolved at  $65^\circ\text{C}$  without denaturing any DNA within it, and will remain fluid at  $37^\circ\text{C}$ . In fact, the manufacturer states that it does not set at temperatures above  $25^\circ\text{C}$ .

The preparative gel was cast as described (section 2.35), using 0.8% (w/v) low melting temperature agarose (BRL), and digested or sonicated DNA was electrophoresed alongside size standards. The gel was

illuminated with long wavelength ultraviolet light, and the desired band was excised with a scalpel. Care was taken not to remove any surrounding agarose not containing the DNA of interest. The gel slice was placed in a pre-weighed 1.5 ml Eppendorf microcentrifuge tube and double distilled water was added in a ratio of 1.5 ml water per gram of agarose.

If non-denatured DNA was required for reaction with restriction enzymes or other DNA modifying enzymes, the agarose was heated to 65°C for 10 minutes, and then held at 37°C.

If denatured DNA was required for use as a probe in Southern hybridisation, the agarose was boiled for 7 minutes and held at 37°C if used immediately for the synthesis of a radiolabelled probe, or stored at -20°C. For subsequent labelling reactions, the stored DNA sample was boiled for 3 minutes and held at 37°C.

## 2.38 Southern Blotting

Three stages are involved: preparation of a [ $^{32}$ P] labelled DNA probe, transfer of DNA fragments separated by electrophoresis to nitrocellulose, and hybridisation of probe DNA to the DNA immobilised on the nitrocellulose.

### 2.38.1 Preparation of [ $^{32}$ P] labelled DNA probe

This was done by denaturing the DNA fragment and labelling with deoxycytidine 5'-[ $\alpha$ - $^{32}$ P] triphosphate ([ $\alpha$ - $^{32}$ P]dCTP) using the large fragment of DNA polymerase I (Klenow fragment), with random pentanucleotides as primers, according to Feinberg and Vogelstein

(1983).

Oligo labelling buffer (OLB) was made by first preparing solutions A, B and C.

- A. 1000  $\mu$ l 1.25 M Tris HCl, 0.125 M  $\text{MgCl}_2$  pH 8.0  
 18  $\mu$ l  $\beta$ -mercaptoethanol  
 5  $\mu$ l 0.1 M dATP (Boehringer Mannheim GmbH) in TE buffer  
 5  $\mu$ l 0.1 M dTTP (Boehringer Mannheim GmbH) in TE buffer  
 5  $\mu$ l 0.1 M dGTP (Boehringer Mannheim GmbH) in TE buffer
- B. 2 M HEPES brought to pH 6.6 with 4 M NaOH.
- C. 50 O.D.<sub>260</sub> units of pentanucleotides (Pharmacia) in 550  $\mu$ l TE buffer to give a concentration of 90 O.D.<sub>260</sub> units  $\text{ml}^{-1}$ .

OLB was 10  $\mu$ l of A, 25  $\mu$ l of B and 15  $\mu$ l of C, mixed together. This was stored at  $-20^\circ\text{C}$ .

The labelling reaction was set up by addition of the following reagents in the stated order:

- 5  $\mu$ l OLB  
 1  $\mu$ l 10  $\text{mg ml}^{-1}$  BSA solution  
 16  $\mu$ l (25 ng) DNA fragment  
 2.5  $\mu$ l [ $\alpha$ - $^{32}\text{P}$ ] dCTP (10  $\text{mCi ml}^{-1}$ , Dupont NEN products)  
 0.5  $\mu$ l Klenow fragment (2 units, Amersham International p.l.c.)

This was incubated at room temperature overnight. The following day,

labelled DNA was separated from unincorporated nucleotides by chromatography through a Sephadex G-50 column equilibrated in TE buffer, using the spun column procedure (Maniatis *et al.*, 1982).

#### 2.38.2 Southern transfer

DNA samples were digested with restriction enzymes and electrophoresed in an agarose gel. The gel, with a ruler alongside it, was photographed on a transilluminator.

Unused areas of gel were trimmed away, and the gel was soaked in 0.25 M HCl at room temperature. This acid depurination partially hydrolyses the DNA and aids transfer of larger DNA fragments. The DNA was then denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl at room temperature for two 15 minute periods.

Transfer of the single stranded DNA fragments to a sheet of nitrocellulose membrane filter (Schleicher and Schuell, BA85) by the capillary blotting method of Southern (1975), was carried out according to Maniatis (1982), with the exception that 20x SSC (standard saline citrate) was used as the transfer buffer. 20x SSC is 3 M NaCl, 0.3 M sodium citrate.

When transfer had proceeded for 12-16 hours, the nitrocellulose was peeled away from the gel and soaked in 6x SSC for 5 minutes. It was then dried at room temperature briefly, and baked for 1-2 hours at 80°C in a vacuum oven. This fixed the DNA to the filter.

The nitrocellulose now had size separated, denatured DNA fragments fixed to it, and could be probed with a labelled fragment of denatured DNA,

which would bind to complementary sequences on the filter.

### 2.38.3 Hybridisation

This was carried out in heat sealable plastic bags. The following stock solutions were prepared.

20x concentrated SSPE contained per litre:

NaCl	174.0 g	i.e. 3 M
Sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ )	27.6 g	
EDTA	7.4 g	

50x concentrated Denhardt's solution contained per litre:

Ficol 400	10 g
Polyvinylpyrrolidone	10 g
BSA	10 g

The stock Denhardt's solution was stored at  $-20^\circ\text{C}$ .

Prehybridisation was carried out by placing the filter in a heat sealable plastic bag (Calor polythene for a Calor bag sealer) with 50 ml of prehybridisation fluid. Prehybridisation fluid was:

5x	SSPE
5x	Denhardt's solution
0.1% (w/v)	SDS

As much air as possible was expelled from the bag, and care was taken to remove bubbles. It was then heat sealed and incubated in a sandwich box submerged in a shaking water bath at  $55^\circ\text{C}$  for 4-8 hours. After this,



the corner of the bag was cut off, the prehybridisation fluid was allowed to drain out and the hybridisation fluid was added to the bag (10 ml for a 132 cm<sup>2</sup> filter). Hybridisation fluid was:

5x	SSPE
5x	Denhardt's solution
0.1% (w/v)	SDS
[ <sup>32</sup> P]	labelled denatured probe DNA
100 $\mu$ l ml <sup>-1</sup>	denatured salmon sperm DNA

The salmon sperm DNA was included to prevent non-specific binding of the very small amount of probe DNA to the nitrocellulose. Both the probe DNA and the salmon sperm DNA were denatured by boiling for 10 minutes followed by immediate transfer to ice for 5 minutes, before being added to the bag. Again, every effort was made to minimise air bubbles as they could prevent efficient contact between filter and hybridisation fluid. The bag was resealed and hybridisation proceeded overnight at 55°C with agitation.

The following day the filter was removed from the bag and washed twice in a solution of 2x SSC, 1x Denhardt's and 0.1% (w/v) SDS for 30 minutes at room temperature. Further washes were carried out, the temperature and salt concentration of which were determined by the stringency required. The Geiger counter was used to check that background radioactivity on the filter was low, before it was air dried, wrapped in cling film and exposed to X-ray film.

### 2.39 Plating Bacteriophage $\lambda$

The procedures used were those in "Molecular Cloning: A Laboratory Manual" (Maniatis et al., 1982).

Briefly, plating bacteria (*E. coli* Y1090) were grown overnight in LB medium supplemented with 0.2% (w/v) maltose. Bacteria grown in the presence of maltose adsorb bacteriophage  $\lambda$  more efficiently than if the maltose were not present, as the sugar induces the maltose operon which contains the gene (*lamb*) for the  $\lambda$  receptor. The bacteria were pelleted by centrifugation at 4500 g for 10 minutes, resuspended in sterile 0.01 M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.4x the volume of the original culture) and stored at 4°C for 0-2 days.

Sterile SM buffer was used for phage storage and dilution, and contained per litre:

NaCl	5.8 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	2.0 g
Tris base	6.05 g and HCl to pH 7.5
Gelatin	0.1 g

50  $\mu\text{l}$  of each phage dilution to be assayed was mixed gently with 100  $\mu\text{l}$  of plating bacteria, and incubated for 20 minutes at 37°C to allow adsorption to occur. Each sample was added to 2.5 ml of LB medium containing 0.7% (w/v) agar or agarose and 10 mM  $\text{MgCl}_2$  in a 12 mm x 75 mm sterile, capped phage dilution tube held at 47°C in a Techam Driblock heated block. The tubes were vortexed gently and poured immediately into a labelled Petri dish containing solid 1.5% (w/v) agar in LB

medium. When the top agar had set, plates were inverted and incubated at the desired temperature (37°C or 42°C).

If a nitrocellulose filter was going to be placed on the bacterial lawn during growth, the plates were poured on a level surface, and agarose was used for the sloppy medium instead of agar which was more likely to adhere to nitrocellulose.

#### 2.40 Assay for Expression of $\beta$ -galactosidase from $\lambda$ gt11

This assay allows determination of the percentage of recombinant and non-recombinant phage in a population of  $\lambda$ gt11. Bacteriophage  $\lambda$ gt11 carries a  $\beta$ -galactosidase gene, and the sole *EcoRI* site on the phage DNA lies within this gene. Functional  $\beta$ -galactosidase cannot be made if DNA has been inserted at the *EcoRI* site. The host bacterium, *E. coli* Y1090, has a deletion which renders it unable to synthesise  $\beta$ -galactosidase. Thus, by plating an appropriate phage dilution series in LB medium containing IPTG (isopropyl  $\beta$ -D-thiogalactopyranoside), an artificial inducer of the *lac* operon, and X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside), a chromogenic substrate which yields a blue colour on  $\beta$ -galactosidase action, an estimate of the percentage of recombinant phage in a  $\lambda$  library can be obtained.

This was done by adding 17  $\mu$ l of a 24 mg ml<sup>-1</sup> solution of IPTG (Sigma) and 50  $\mu$ l of a 20 mg ml<sup>-1</sup> solution of X-gal (Sigma) in dimethylformamide to each 2.5 ml of top agarose before the bacteria and phage were added, then plating as described.

#### 2.41 Immunological Screening of a $\lambda$ Library

$\lambda$  DNA was packaged using a lambda *in vitro* packaging kit (Amersham International p.l.c.) according to the manufacturer's instructions. The library was plated as described, and the plates were incubated at 42°C for 3.5 hours. At this stage, the plates were removed from the incubator and overlaid with a dry 82 mm diameter disc shaped nitrocellulose membrane filter which had been saturated in 10 mM IPTG. The plates were returned to the incubator and incubated at 37°C for a further 3.5 hours, during which time the IPTG induced transcription from the promoter of the  $\beta$ -galactosidase gene (*lacZ*), resulting in expression of  $\beta$ -galactosidase from non-recombinant phage, and fusion proteins from recombinant phage. The plates were then removed to room temperature, the position of the filter on the agar was marked with a needle, and each filter was transferred to a clean Petri dish containing 25 ml 3% (w/v) BSA in PBS.

The system used for detecting antibody bound to protein produced by phage particles in a plaque, employed biotinylated Protein A and streptavidin biotinylated horseradish peroxidase complex (both from Amersham International p.l.c.). The protocol was a scaled down version of that in section 2.25.3, which was followed exactly, except that the volumes of all the solutions used were halved, and it was carried out in Petri dishes rather than sandwich boxes.

#### 2.42 Transposon Mutagenesis

The donor and recipient strains were brought into proximity on a

membrane filter, and mating proceeded overnight.

The *Rm. vannielii* recipient strain was grown to mid exponential phase, anaerobically in the light in PM medium containing  $100 \mu\text{g ml}^{-1}$  nalidixic acid. To coincide with this, the *E. coli* donor strain was grown overnight in LB medium containing the appropriate antibiotic, in a static universal on its side at an angle of approximately  $10^\circ$ , at  $37^\circ\text{C}$ . The recipient and donor cultures, in a ratio of 10:1 by volume, were carefully transferred to a sterile universal. They were mixed gently by inversion and applied to a sterile  $0.22 \mu\text{m}$  pore size, 47 mm diameter MF-Millipore filter housed in a Millipore Sterifil aseptic system (47 mm diameter) which consisted of a funnel with a cover, a filter holder and a 250 ml receiver flask connected to a vacuum pump (the Millipore apparatus and filters had been sterilised by autoclaving at 10 p.s.i. for 10 minutes). The filtration resulted in the cells being concentrated on the filter, and they were washed with 10 ml of sterile PM medium to remove the antibiotics. The filter was then aseptically removed from the apparatus and placed on a PMY agar plate, which was inverted and incubated at  $30^\circ\text{C}$  aerobically in the dark overnight.

The following day, the filter was placed in a sterile universal containing 2-4 ml of sterile PM medium. Cells were washed off the filter and resuspended by vortexing. This suspension was concentrated by centrifugation at 4500 g for 20 minutes, if necessary. 0.1 ml was then spread on PMY agar plates containing antibiotics which selected (a) against the donor strain and (b) for those *Rm. vannielii* cells expressing the drug resistance determinant carried on the transposon. The plates were allowed to dry, and were incubated microaerophilically in the dark for 14 days at  $30^\circ\text{C}$  as described in section 2.4.

#### 2.43 Sequence Comparisons

Comparisons of published flagellin sequences were made using the EMBL (European Molecular Biology Laboratory) database.

CHAPTER 3

PROTEINS ASSOCIATED WITH MOTILITY IN

*Rhodomicrobium vannielii*

### 3 PROTEINS ASSOCIATED WITH MOTILITY IN *Rhodocrobium vannielii*

For reasons described in section 1.1, i.e. in order to examine differential expression of specific proteins it was necessary to identify some of the proteins associated with the periodic motility exhibited by *Rm. vannielii* during its cell cycle, and this was the starting point of this study.

#### 3.1 Characterisation of CsCl Purified Flagella

Flagella from *Rm. vannielii* which had been shed into the medium were concentrated using an Amicon hollow fibre concentrator and purified by caesium chloride density gradient centrifugation (section 2.14). The flagella formed a thick white band at a density of  $1.336 \text{ g cm}^{-3}$ .

If a sample of this material was viewed in the light microscope before dialysis, the long helical bundles of flagella shown in Fig. 3.1 could be seen. Similar bundles of flagella from *Caulobacter crescentus* culture supernatants have been observed (Poindexter *et al.*, 1967).

After the removal of the caesium chloride by dialysis, samples of the material taken from the gradient were stained with 1% (w/v) phosphotungstic acid and examined in the electron microscope (section 2.9). A mass of flagellar filaments was observed, an example of which is shown in Fig. 3.2. Sometimes the length and waveform of the filament was preserved, and with other samples shorter, curved lengths of filament could be seen. It was difficult to observe any other features of flagella, e.g. hooks, in samples of flagella prepared using the





Fig. 3.1 Light micrograph of helical bundles of flagella from *Rm. vannielii* at 1000x magnification. A *Rm. vannielii* cell (2-3  $\mu$ m in length) is included in the centre of the micrograph to give an idea of the large size of the flagella bundles.



Fig. 3.2 Electron micrograph of *Rm. vannielli* flagellar filaments.

Bar = 0.2  $\mu$ m. —

Amicon hollow fibre concentrator and CsCl gradient centrifugation.

SDS polyacrylamide gel electrophoresis was used to characterise the proteins present in samples of flagella purified in this way. When such a gel was stained with Coomassie blue (Fig. 3.3(a)), only one protein band was visible. This had a molecular weight of 34k, and was the filament protein, flagellin.

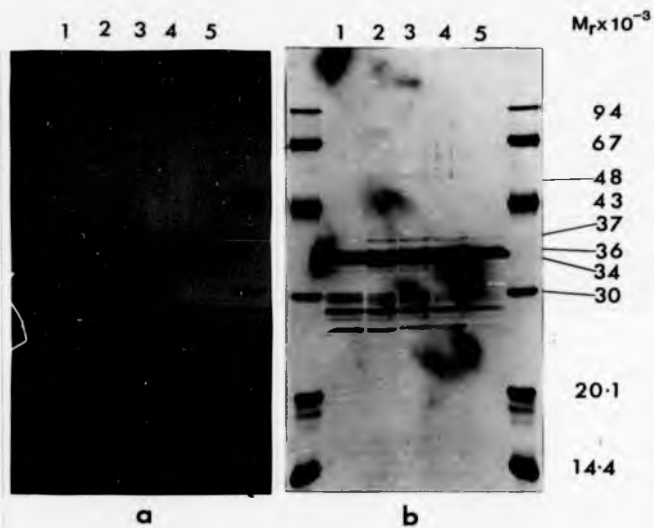
When the same gel was silver stained, it was seen to be overloaded with respect to flagellin, but some other proteins were visible. These were a 37k protein, a very faintly visible 48k protein and several bands of 30k or less. The fact that the 37k protein and the 48k protein are not visible in track 1, where the sample was not heated prior to loading, but were visible in track 2 where the same sample was boiled for 3 minutes in SDS sample buffer (section 2.16), suggested that these two proteins were associated with the flagellar filament.

Evidence from work with antisera raised against this and other flagella preparations shed more light on the identity of these proteins. It suggested that the bands of 30k and less represented flagellin polypeptides which have in some way been degraded during preparation. It should be borne in mind that, for silver staining, the gel is very heavily loaded with protein, and the minor proteins mentioned above are probably present in very small amounts.

4 antisera (numbered 1-4) were raised against *Rm. vannielii* flagella or components thereof. The rabbits were inoculated (section 2.22) with the samples described below.

Fig. 3.3 SDS 10% (w/v) polyacrylamide gel electrophoresis of *Rm. vannielii* flagella concentrated from culture medium using an Amicon concentrator and purified on a CsCl gradient. The tracks contain:

1. 12  $\mu$ g protein, not heated.
2. 12  $\mu$ g protein, boiled for 3 minutes in SDS sample buffer.
3. 9  $\mu$ g protein, boiled for 3 minutes in SDS sample buffer.
4. 6  $\mu$ g protein, boiled for 3 minutes in SDS sample buffer.
5. 3  $\mu$ g protein, boiled for 3 minutes in SDS sample buffer.



Antiserum    Sample used to inoculate rabbit

- |     |  |
|-----|--|
| 1 } | 2 separate preparations of   |
| 2 } | CsCl purified flagella   |
| 3   | Flagella purified through 2 CsCl gradients   |
| 4   | The 34-36k band of protein purified from a polyacrylamide gel by electroelution (section 2.21).<br>The preparative gel was loaded with CsCl purified flagella. |

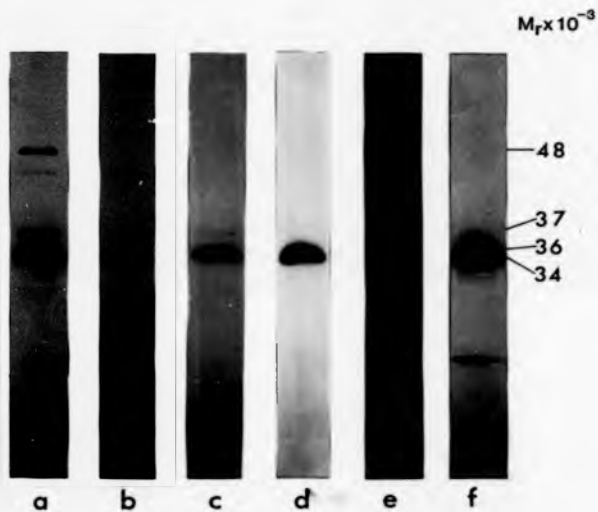
Western blotting was used to examine the binding specificity of these antisera to protein in cell free extracts of *Rm. vannielii*, and thus to characterise further the flagellar proteins. Cell free extract was prepared from a late exponential phase batch culture of *Rm. vannielii* (section 2.11), and subjected to SDS polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose and probed with the 4 antisera and 4 corresponding pre-immune sera. Radioiodinated protein A was used to visualise the protein bands which bound antibodies (Fig. 3.4).

No bands appeared on autoradiograms of blots performed with the pre-immune sera. Fig. 3.4(d) shows that the electrophoretic protein purification successfully yielded an antiserum which reacted only with proteins in the 34-36k molecular weight region of a *Rm. vannielii* cell free extract. The prior knowledge that the *Rm. vannielii* hook protein has a molecular weight of 36k (section 3.2), i.e. close to that of flagellin, is useful in interpreting these results.

The 48k protein was only lit up by one of the four antisera described (1, Fig. 3.4(a)). It is probably an outer membrane or inner membrane

Fig. 3.4 Western blots of *Rm. vannielii* cell free extract, prepared from a batch culture, 75  $\mu$ g of protein per track), probed with the following antisera:

- (a) Antiserum 1, 36 hour exposure to X-ray film.
- (b) Antiserum 2, 36 hour exposure to X-ray film.
- (c) Antiserum 3, 36 hour exposure to X-ray film.
- (d) Antiserum 4, 36 hour exposure to X-ray film.
- (e) Antiserum 3, 4 week exposure to X-ray film.
- (f) Antiserum 4, 4 week exposure to X-ray film.





protein copurifying with flagella rather than an integral part of the shed flagella. This does not rule out the possibility that it may have a function associated with motility.

However, the 37k protein seems to be genuinely associated with shed flagella, as it appeared in all the blots except the one probed with antiserum raised to gel purified proteins. In *C. crescentus*, filament, hook and rod are shed into the medium. The rods are most clearly visible in electron micrographs of hook and rod structures shed into the medium by a mutant which does not assemble filament, but sheds hook and rod with normal periodicity (Johnson *et al.*, 1979). On examination of electron micrographs of hook and filament shed by *Rm. vannielii* (Fig. 3.5), it was not possible to see a slight narrowing at the cell proximal end of the hook in Fig. 3.5(b). However, assuming that filament and hook are made up of one type of polypeptide subunit each, one candidate for the identity of the 37k protein seems to be rod protein.

The 37k protein can also be seen in Fig. 3.7, a gel showing fractions from a sucrose gradient used to isolate hooks. It appeared in fractions 2-4, which were the ones which contained the greatest amount of hook (with some filament) in the electron microscope. Ikeda *et al.* (1985) discovered the presence of a "cap" structure at the tip of *S. typhimurium* flagella which was never observed at the tip of flagellar filaments assembled *in vitro* from flagellin monomers. Recently immunogold labelling techniques have provided evidence that the cap is made from a polypeptide

previously designated hook-associated protein 2 (Ikeda *et al.*, 1987). This study described the localisation of two other hook-associated proteins, and it is possible that the 37k protein from *Rm. vannielii* is a cap or hook-associated protein. The cap is thought to play an essential role in the flagellar elongation mechanism *in vivo*, and intact filaments have been found to be unable to serve as nucleation centres for the assembly of exogenous flagellin *in vitro*, probably due to the presence of the cap (Ikeda *et al.*, 1985).

Hook protein represents about 1% of the mass of the bacterial flagellum in *E. coli* and *S. cyphimurium* (Silverman and Simon, 1977), and the *Rm. vannielii* hook band is not visible as a discrete band in Western blots, probably due to merging with the strong signal given out by the  $^{125}\text{I}$  bound to flagellin. This explains why a broad band in the 34-36k region appeared in blots using each of the 4 antisera. Also, there is evidence

that flagella prepared using the hollow fibre concentrator and CsCl gradients have very little hook associated with them, from electron microscopy and the fact that it was difficult to isolate hooks from flagella prepared in this way. For these reasons flagellin and hook protein were not resolved in the silver stained gel in Fig. 3.3, and it was only possible to identify hook protein when the ratio of hook protein to flagellin was increased by enriching for hooks and excluding much of the filament, as described in the next section.

### 3.2 Identification of Hook Protein

Flagellar hooks are much more stable to acid dissociation than flagellar filaments. This can be exploited to enrich for hooks from a preparation of shed flagella by lowering the pH to 3.5, and collecting hooks by centrifugation (section 2.15).

The method of preparation of starting material proved to be important for the successful isolation of hooks. If shed flagella were concentrated from the medium using the Amicon hollow fibre concentrator, no hooks could be observed in the electron microscope, and at the end of the isolation procedure, no protein other than flagellin could be detected on a silver stained gel. However, if flagella were ammonium sulphate precipitated from the culture medium and collected by centrifugation (section 2.14), the isolation of hooks was successful.

This suggests that the white band removed from CsCl gradients containing shed flagella which had been concentrated using the Amicon concentrator consisted predominantly of filament, which was supported by electron

microscopy of such samples. However, there was enough hook protein present in these preparations to elicit an antibody response, as discrete 34k and 36k bands could be seen in some Western blots (not shown). This is not incompatible with the above observation, as extremely small amounts of protein will stimulate an antibody response if the protein is immunogenic, and the amount of antibody synthesised is not proportional to the amount of protein inoculated into the animal. It appears that the hook and filament were sheared during the Amicon concentration step, which involved the removal of water and other molecules with molecular weights less than 100k from the sample under pressure. If shearing did occur during this step, then filaments would band at their own buoyant density in the subsequent CaCl density gradient centrifugation, while hooks would band elsewhere.

Therefore, the pH of a preparation of shed flagella collected by  $(\text{NH}_4)_2\text{SO}_4$  precipitation, was lowered to 3.5 by dropwise addition of glacial acetic acid. Electron microscopy showed that this treatment effected complete dissociation of flagellar filaments. After high speed centrifugation of the acidic solution, the pellet, which was pigmented, was resuspended in a small volume of buffer at pH 8. When a sample of this was examined in the electron microscope, the most common structure visible was a hook with a short length of filament attached. This was taken to indicate that hooks or hooks plus a very short piece of filament act as nucleation sites for the reassembly of flagellin subunits into filaments upon return to pH 8. Similar *in vitro* reassembly of filaments from flagellin subunits has been observed with *Salmonella typhimurium* (Kagawa *et al.*, 1973) and *Caulobacter crescentus* (Sheffery and Newton, 1977).

At this stage, the pigmented preparation was subjected to sucrose density gradient centrifugation as used by Lagenaur *et al.* (1978), and described in section 2.15, in an attempt to concentrate and purify hooks. The gradient was fractionated from the bottom, and a sample from each fraction was run on a denaturing polyacrylamide gel (Fig. 3.6). After dialysis to remove the sucrose, the fractions were also examined in the electron microscope.

The electron microscopy showed that fractions 2 and 3 were much enriched for hooks (with short lengths of filament attached), and electron micrographs of samples from these fractions are shown in Fig. 3.5.

From the gel it can be seen that the predominant protein, other than flagellin, in fractions 2 and 3 is a 36k polypeptide, which was designated hook protein. The gel also showed that hooks did not concentrate at a specific buoyant density, which may have been due to overloading of the gradient. However, the pigmented material was separated from the hooks by the sucrose gradient centrifugation, as only fractions 14, 15 and 16 contained pigment.

### 3.3 *Em. vannielii* Anti-Flagella Antiserum Cross-Reactivity With Proteins From Other Members of the Rhodospirillaceae and *C. crescentus*

This experiment was performed partly to assess amino acid sequence homology between *Em. vannielii* and *Caulobacter* flagellins, and partly to obtain an indication of whether flagellin structure is conserved among the purple non-sulphur bacteria.



a

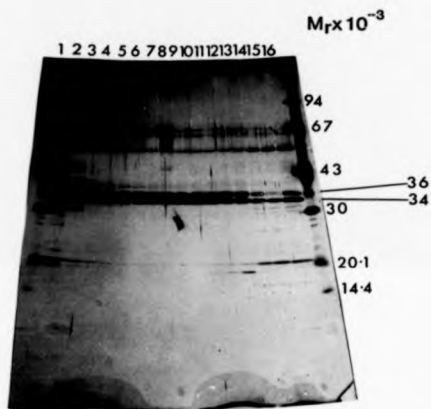


b

Fig. 3.5 Electron micrographs of *Rm. vannielii* flagellar hooks.

Bar = 0.2  $\mu$ m. —

Fig. 3.6 SDS 10-30% (w/v) polyacrylamide gel electrophoresis of 60  $\mu$ l samples of fractions from a sucrose gradient loaded with *Rm. vannielii* flagellar hooks isolated from  $(\text{NH}_4)_2\text{SO}_4$  precipitated, shed flagella. Track 1 corresponds to the bottom of the gradient, while track 16 represents the top.





Cell free extracts were prepared, as in section 2.11, from late exponential phase cultures of *Caulobacter crescentus* and the members of the *Rhodospirillaceae* listed in Table 3.1. Although the volumes of cell free extract loaded on an SDS polyacrylamide gel were adjusted such that each track contained an equal amount of protein in total, (75  $\mu$ g), the amounts of flagellin and hook protein loaded could not be considered equal, as the percentage of cells which were motile in cultures of the different species varied considerably. An estimate of the number of motile cells in a late exponential phase culture, as judged by light microscopy, is given in Table 3.1. The estimates are given relative to the proportion of motile cells in a late exponential phase culture of *Rm. vannielii*, which for this purpose is defined as +++.

Proteins from these cell free extracts were separated in a denaturing polyacrylamide gel and probed with antiserum 4 (Fig. 3.4(d) and (f)) by Western blotting. After a 36 hour exposure to X-ray film, only two bands could be seen; one in the *Rm. vannielii* track (track 10) in the 34-36k region, and one in the *C. crescentus* track (track 8) with a molecular weight of approximately 26k. These are the bands marked with arrows in Fig. 3.7.

This result was surprising, as it indicates that *Rm. vannielii* flagellin is more closely related to *C. crescentus* flagellin than to flagellins from other members of the *Rhodospirillaceae*.

When the same blot was exposed to X-ray film for 4 weeks, the autoradiogram shown in Fig. 3.7 was the result. A 70k band was now visible in the *C. crescentus* track (track 8), indicating that antibodies raised against *Rm. vannielii* flagellin and hook cross react with

Table 3.1 Motility in cultures of *C. crescentus* and members  
of the Rhodospirillaceae

Bacterium	Indication of proportion of motile cells in late exponential phase culture relative to that in <i>Rm. vannielii</i>
<i>Rhodomicrobium vannielii</i>	+++
<i>Rhodobacter sphaeroides</i>	++
<i>Rhodobacter capsulatus</i>	++
<i>Rhodopseudomonas viridis</i>	+++
<i>Rhodopseudomonas palustris</i>	+++
<i>Rhodocyclus gelatinosus</i>	+
<i>Rhodopseudomonas acidophila</i>	+
<i>Rhodopseudomonas blastica</i>	NON MOTILE
<i>Caulobacter crescentus</i>	+++
<i>Rhodospirillum rubrum</i>	++++

Fig. 3.7 Western blot of cell free extracts (75  $\mu$ g of protein per track) prepared from various species from the *Rhodospirillaceae* and *Caulobacter crescentus*, probed with antiserum 4. Exposure to X-ray film was for 4 weeks. The tracks contained cell free extracts from the following bacteria:

1. *Rhodobacter sphaeroides*
2. *Rhodobacter capsulatus*
3. *Rhodopseudomonas viridis*
4. *Rhodopseudomonas palustris*
5. *Rhodocyclus gelatinosus*
6. *Rhodopseudomonas acidophila*
7. *Rhodopseudomonas blastica*
8. *Caulobacter crescentus*
9. *Rhodospirillum rubrum*
10. *Rhodomicrobium vannielii*

$M_r \times 10^{-3}$ 

*C. crescentus* flagellin and hook. Also in Fig. 3.7 it can be seen that, eventually, one or two bands appear in every track, with the exception of track 7, which contains cell free extract from the non-motile *Rhodospseudomonas blautica* as a negative control. It is probable that these bands are flagellins and hook proteins from the other bacteria, but this conclusion should be treated with caution, as bands which are not flagellin or hook from *Rm. vannielii* do light up (track 10 in Fig. 3.7 and Fig. 3.4(f)) after such long exposure.

Flagellins from different bacteria vary considerably in molecular weight and antigenicity, and it is generally held that flagellins are not well conserved among genera, or among different species within a genus. There is evidence that hook proteins are more strongly conserved than flagellins (Kagawa *et al.*, 1973; Sheffery and Newton, 1979).

Amino acid sequence comparisons made by Joys (1985) showed some homology at the N- and C-terminus of flagellins from *Salmonella typhimurium*, *Bacillus subtilis* and *Caulobacter crescentus*. The three flagellins varied considerably in molecular weight. Within the genus *Salmonella*, he showed that both ends of the flagellin molecule have conserved amino acid sequences, while the central region is variable.

These comparisons show that there are precedents for structural homologies between flagellins of bacteria from different genera, and suggest that certain immunoglobulin molecules from a population of polyclonal antibodies directed against *Rm. vannielii* flagellin could well bind to the N- or C-terminus of *C. crescentus* flagellins.

However, in the enteric bacteria, the variation among flagellin

antigenicity is so great that serological cross-reactivity does not exist between different strains of the same species in some cases. Also, in *Salmonella typhimurium*, one bacterial cell has the ability to alter its flagellar antigen between two different types (section 1.2.2.1). These observations have been attributed to the need of these organisms to evade the mammalian immune response (Kagawa et al., 1973; Silverman and Simon, 1983).

There is no obvious comparable selective pressure on the *Rhodospirillaceae* to have antigenically diverse flagellins, with the possible exception of avoiding bacteriophage infection, but the results of Western blotting using anti-*Rm. vannielii* flagellin and hook antiserum suggest that considerable antigenic variation does exist. It seems likely that the converse argument applies, and that the diversity arose because there was no environmental pressure to maintain amino acid sequence homogeneity, especially in the central region of the protein.

Flagellin DNA sequence homologies are discussed in sections 5.1 and 5.1.1.

### 3.4 Methyl-Accepting Chemotaxis Proteins

As the study of methyl-accepting chemotaxis proteins in *C. crescentus* was very fruitful with regard to information on timed gene expression and localisation of specific gene products to a particular cell type at cell division (sections 1.5.2.1 and 1.5.3.1), the detection of MCPs in *Rm. vannielii* was attempted.

Fig. 3.8 Fluorogram of SDS 10% (w/v) polyacrylamide gel containing samples of proteins from cells labelled with [methyl-<sup>3</sup>H] methionine. The contents of the tracks are:

1. 105  $\mu$ g of protein from a *Rm. vannielii* batch culture labelled with 75  $\mu$ Ci ml<sup>-1</sup> [methyl-<sup>3</sup>H] methionine.
2. 105  $\mu$ g of protein from a *Rm. vannielii* swarmer cell population labelled with 15  $\mu$ Ci ml<sup>-1</sup> [methyl-<sup>3</sup>H] methionine.
3. 105  $\mu$ g of protein from a *C. crescentus* batch culture labelled with 15  $\mu$ Ci ml<sup>-1</sup> [methyl-<sup>3</sup>H] methionine.
4. 255  $\mu$ g of protein from the sample used in track 3.
5. 255  $\mu$ g of protein from the sample used in track 2.

The dried gel was exposed to X-ray film for one week.

This was done, as described in section 2.27 and Shaw *et al.* (1983), by stopping the elongation of polypeptide chains, and thus the incorporation of labelled methionine into protein, with chloramphenicol, and adding [methyl- $^3\text{H}$ ] methionine to an exponential phase culture. Under these conditions, any labelled proteins detected in an SDS polyacrylamide gel have been covalently methylated by enzymatic transfer of a tritiated methyl group to the protein.

The optimal concentration of chloramphenicol and time of incubation to prevent incorporation of [ $^{35}\text{S}$ ] methionine into protein in *Rm. vanniellii* were found to be the same as those used by Shaw *et al.* with *C. crescentus*:  $50 \mu\text{g ml}^{-1}$  for 5 minutes.

Despite labelling heterogeneous cultures with much higher concentrations of [methyl- $^3\text{H}$ ] methionine than those used for *C. crescentus*, and labelling homogeneous populations of swarmer cells, in an effort to increase the specific activity of any sensory transduction enzymes, no methylated proteins were detected in *Rm. vanniellii* (see Fig. 3.8).

The *C. crescentus* controls (tracks 3 and 4) showed that the *Caulobacter* MCPs were methylated with [ $^3\text{H}$ ] methyl groups under these experimental conditions. Equal amounts of protein were loaded in tracks 1-3 and in tracks 4 and 5 (see legend to Fig. 3.8).

From this, it can be concluded that *Rm. vanniellii*, like *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* (Sackett *et al.*, 1987) does not have MCPs which can be detected using <sup>the</sup> *in vivo* labelling technique which has shown the presence of MCPs in the enteric bacteria and *C. crescentus*. However, it has recently been shown that it is necessary to

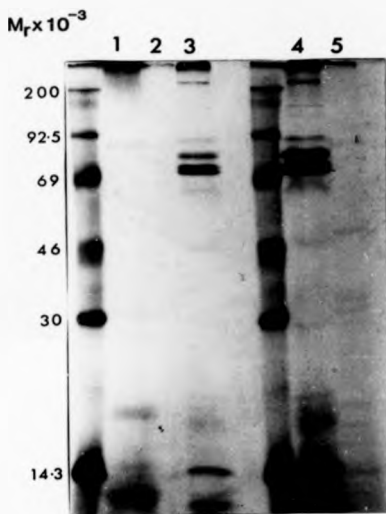


use other techniques, such as (i) *in vitro* methylation using washed membranes, cytoplasmic extracts and S-[adenosyl-<sup>3</sup>H] methionine and (ii) assaying for the release of [<sup>3</sup>H] methanol from prelabelled MCPs upon removal of an attractant, to detect MCPs in the *Rhodospirillaceae* (Sackett *et al.*, 1987). Although methylated proteins were not detected in *Rhodobacter sphaeroides* or *Rhodospirillum rubrum* using *in vivo* labelling, MCPs could be detected in *Rsp. rubrum* when *in vitro* methylation studies and methanol production assays were carried out. MCPs were not detected in *R. sphaeroides* using any of these techniques (Sackett *et al.*, 1987).

Thus, although the existence of MCPs in *R. sphaeroides* has been ruled out, *in vitro* methylation studies and methanol production assays must be performed with *Rm. vannielii* before definite conclusions about the presence or absence of MCPs can be drawn. Another improvement would be to use *Rm. vannielii* cells which have been stimulated by the addition of an attractant.

Although MCPs were detected in *Rsp. rubrum*, Sackett *et al.* (1987) point out that the MCPs are probably not used for the major chemoeffectors for the *Rhodospirillaceae*, such as propionate, since these are attractants for both *Rsp. rubrum* and *R. sphaeroides*. They suggest that *Rsp. rubrum* probably uses several different chemosensory pathways, including those involving transport (Ingham and Armitage, 1987), with only a few chemicals being sensed via MCPs.

Several differences between the motility exhibited by *R. sphaeroides* and



that exhibited by the enteric bacteria have been characterised by Armitage and Macnab (1987). *R. sphaeroides* is unusual structurally in that its single flagellum is positioned medially, i.e. it arises approximately halfway along the long axis of the cell, and it has an unusual narrow, straight hook (Sackett and Armitage, 1986). It also differs functionally from the enteric bacteria. Hitherto, such features as switching direction of rotation of flagella to reorient the bacterium, and signal transduction via MCPs were accepted as general features of bacterial motility. However, not only does *R. sphaeroides* not use MCP methylation, it swims and changes direction rotating its flagellum in the clockwise direction only, and reorients itself by briefly stopping flagellar rotation and allowing Brownian motion to reorient the cell.

Therefore, how does *R. sphaeroides* carry out chemotaxis? Two suggestions have been made:

(i) Armitage and Macnab (1987) have suggested that *R. sphaeroides* may only respond to environmental changes which result in a change in proton motive force (see section 1.3.3). This is based on the observations that tactic stimulation results in a change in membrane potential in *R. sphaeroides*, but not in *E. coli*, and that addition of low levels of uncouplers to reduce the electrochemical proton gradient inhibits the tactic response in *R. sphaeroides* (Sackett and Armitage, 1986).

(ii) Taylor *et al.* (1983) remarked that a common feature of chemotaxis receptors is that they serve a dual role as receptors for both chemotaxis and active transport. It has been observed with *R. sphaeroides* that in the presence of high concentrations of a

chemoattractant, chemotaxis does not take place, whereas in low concentrations of the chemoattractant chemotaxis occurs. This led to the proposal that it may be only when a protein for the uptake of a particular chemoattractant is induced, e.g. when the chemoattractant is present in low concentrations, that chemotaxis towards that chemoattractant occurs (J. P. Armitage, personal communication and Ingham and Armitage, 1987).

It is possible that membrane proteins involved in the transport of a chemoattractant across the cytoplasmic membrane are chemoreceptors in *R. sphaeroides*, as the enzymes II of the phosphotransferase system are in *E. coli* (section 1.3.2). That this *E. coli* chemosensory transduction is mediated by reversible phosphorylation and dephosphorylation of membrane proteins at histidine residues has been suggested by Langeler *et al.* (1981). If such a system does exist in the *Rhodospirillaceae*, it is interesting to note that, in addition to phosphoserine, phosphothreonine and phosphotyrosine, Turner and Mann (1986) found another unidentified phosphorylated residue in *Rm. vannielii* protein hydrolysates, and to speculate that this could be phosphohistidine.

However, how similar *R. sphaeroides* and *Rm. vannielii* turn out to be with respect to motility and chemotaxis remains to be seen. In preliminary experiments, *Rm. vannielii* swarmer cells were tethered to a microscope slide using a 1:1000 dilution of anti-flagella antiserum and 7 tethered cells were observed for approximately a minute each. In 3 cases the cell body rotated counter clockwise with stops, and in 4 cases the cell body rotated predominantly counter clockwise, but stopped, changed direction, and rotated clockwise for part of the time. The number of cells examined was very small, but *Rm. vannielii* swarmer cells are capable of bidirectional flagellar rotation. As with *R. sphaeroides*

and other bacteria, speed of rotation varied from cell to cell.

The relationship between tactic stimulation and membrane potential in *Rm. vannielii* has not been investigated, but membrane potential has been measured in this organism (Kelly, 1985), and this has yielded information on (i) envelope permeability during the cell cycle and (ii) differences in the light-induced increase in membrane potential in swarmer cells and reproductive cells.

Regarding (i): *Rm. vannielii* was not permeable to the permeant ion, tetraphenylphosphonium ( $\text{TPP}^+$ ), even in the presence of EDTA, which is known to permeabilise the outer membrane of *E. coli* (Nikaido and Vaara, 1985), so  $\text{TPP}^+$  could not be used to measure membrane potential. In contrast, *R. sphaeroides* was permeable to  $\text{TPP}^+$  in the presence of EDTA, allowing measurement of changes in membrane potential. Additionally, after it was found that membrane potential in *Rm. vannielii* could be measured using the carotenoid bandshift, it was shown that the uncoupler FCCP collapsed the membrane potential much more quickly in reproductive cells than swarmer cells (Kelly, 1985), which is in agreement with the evidence (section 1.6.2.3) that the reproductive cell envelope of *Rm. vannielii* is more permeable than the swarmer cell envelope.

The data concerning point (ii) were obtained by making use of the electrochromic response of endogenous carotenoids (changes in the carotenoid bandshift) to measure light-induced membrane potential in *Rm. vannielii*. Using synchronised, differentiating swarmer cell populations it was found that the value of the light-induced membrane potential decreased during swarmer cell differentiation (Kelly, 1985).

In conclusion, now that motility and chemotaxis are being investigated in a more diverse range of bacteria, the role of membrane potential in tactive responses of photosynthetic bacteria may become clearer. Moreover, it may become possible to assess how widespread the "enteric type" of motility is, and understand how motility and chemotaxis operate in groups of bacteria which are found in different environments, and differ physiologically.

### 3.5 Isolation of Basal Bodies from *Rm. vannielii*

Attempts to isolate flagellar basal bodies from *Rm. vannielii* were unsuccessful due to the difficulty in achieving gentle lysis of the bacterium. As yet, gentle lysis is only possible at growth points (Dov *et al.*, 1983).

A combination of the procedures of DePamphilis and Adler (1971a) and Cohen-Bazire and London (1967) resulted in extremely poor lysis of *Rm. vannielii* cells, and the small pellet obtained from high speed centrifugation after removal of whole cells did not contain any basal bodies as judged by electron microscopy. The procedures currently in use with *Caulobacter crescentus* are described in Hahnenberger and Shapiro (1987).

### 3.6 Summary

1. Shed flagella from *Rm. vannielii* consisted of 3 proteins detectable on silver stained denaturing polyacrylamide gels:

Flagellin ( $M_r$  34k), hook protein ( $M_r$  36k) and a protein of  $M_r$  37k which may be rod protein or a minor protein associated with flagella.

2. Antiserum raised against *Rm. vannielii* shed flagella cross reacted with *Caulobacter crescentus* flagellin and hook protein and, to a significantly lesser extent, with proteins from motile members of the *Rhodospirillaceae*.
3. No methyl-accepting chemotaxis proteins were detected in *Rm. vannielii* using the *in vivo* labelling technique of Shaw *et al.* (1983).

CHAPTER 4

PERIODIC SYNTHESIS OF FLAGELLIN



4 PERIODIC SYNTHESIS OF FLAGELLIN

The aim of these experiments was to establish the period of flagellin synthesis during the *Rm. vannielii* cell cycle.

In an early experiment, samples were removed at hourly intervals from a synchronised *Rm. vannielii* population, and unlabelled cell free extracts were prepared, prior to denaturing polyacrylamide gel electrophoresis. The gel was blotted onto nitrocellulose and probed with anti-flagella antiserum, to examine changes in the amount of flagellin present as the swarmer cells differentiated into non-motile reproductive cells.

As Western blotting is extremely sensitive, flagellin was detected throughout differentiation (data not shown). This may have been due to the presence of some shed flagella which were not removed when the cells were washed, or flagellin synthesised by a small number of unsynchronised swarmer cells present in the reproductive cell samples. It was concluded that as it was difficult to assay for the presence or absence of flagellin during the cell cycle, it was necessary to look at synthesis of the flagellin protein during well defined periods of the cycle. This approach allowed the determination of the period of flagellin synthesis during differentiation in *Rm. vannielii*.

Samples were removed from a synchronised population of cells at hourly intervals and pulse-labelled with [ $^{35}$ S] methionine. (section 2.10). Cell free extracts were prepared from the radiolabelled samples (section 2.11) and each was immunoprecipitated with anti-flagella antiserum (section 2.26).

Fig. 4.1 shows a fluorogram of the immunoprecipitates from the hourly

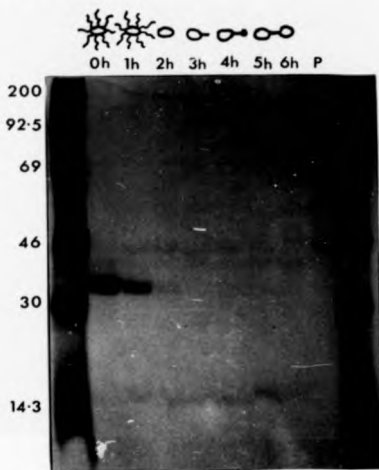
samples run on an SDS polyacrylamide gel. Volumes of cell free extract were adjusted so that an equal number of counts per minute was added to the immunoprecipitation reaction for each sample. The track labelled "P" contains the same number of counts per minute from the 0 h sample precipitated with pre-immune serum, and no labelled proteins were visible.

The appearance of the cells in the light microscope during the experiment is shown above each track in Fig. 4.1, and the cell volume distribution profiles of the cells at four of the sampling times during the experiment are shown in Fig. 4.2.

From Fig. 4.1 it can be seen that in swarmer cells incubated anaerobically in the light ( $40 \mu\text{Em}^{-2}\text{s}^{-1}$ ), flagellin is synthesised for one hour, but is switched off upon initiation of differentiation.

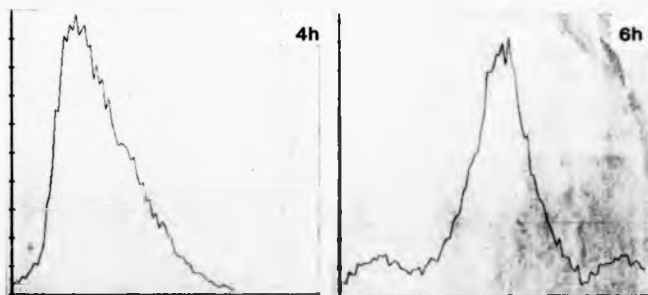
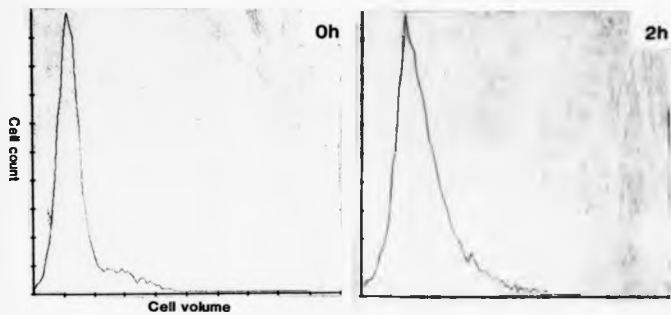
The cell volume distribution profiles (Fig. 4.2) show a sharp peak at the swarmer cell volume for the 0 h sample, indicating that the synchronisation procedure yielded a homogeneous population of swarmer cells. Cell volume increases as stalk and bud form, until, by the 6 h sample, the profile shows that the cell volume peak has moved along the x-axis to that volume representing a pair of mother and daughter cells, i.e. the population has differentiated synchronously.

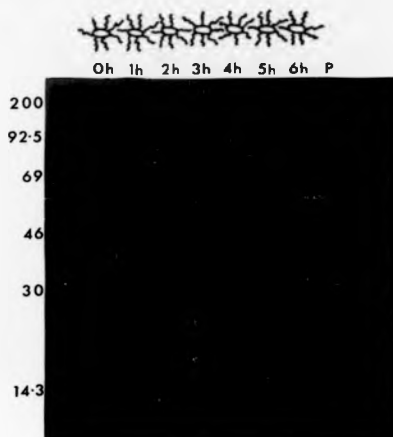
Fig. 4.3 shows the result if the radioimmunoprecipitation of hourly samples is repeated, with the difference that the synchronised swarmer cells are held anaerobically in the dark for six hours, at the same temperature as previously ( $30^\circ\text{C}$ ). Flagellin is synthesised throughout the six hours.

$M_r \times 10^{-3}$ Fig. 4.1

Fluorogram of radioimmunoprecipitates prepared from samples removed hourly from a synchronised population of differentiating *Rm. vannielii* cells incubated anaerobically in the light. Cell free extract containing 800,000 c.p.m. was immunoprecipitated for each sample. Autoradiogram exposure was one week.

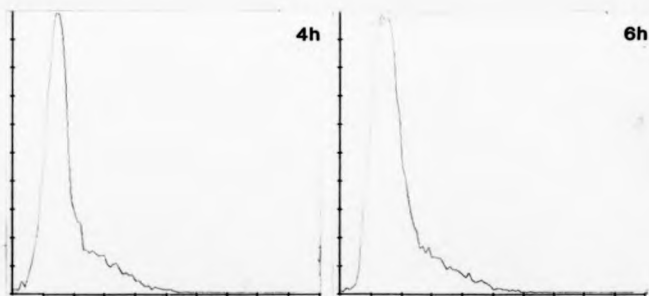
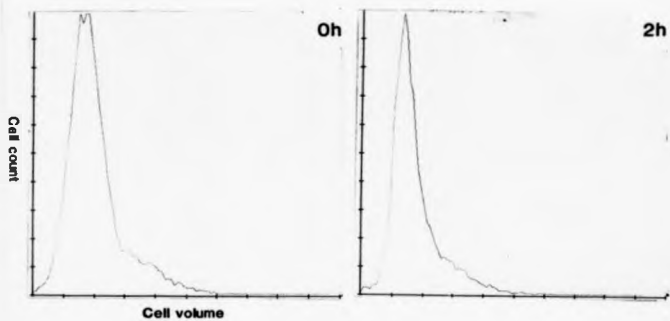
Fig. 4.2 Cell volume distribution profiles (obtained as described in section 2.7) of samples removed from a synchronised population of differentiating *Rm. vannielii* cells after 0 hours, 2 hours, 4 hours and 6 hours anaerobic incubation in the light.



$M_r \times 10^{-3}$ 


**Fig. 4.3** Fluorogram of radioimmunoprecipitates prepared from samples removed hourly from a synchronised population of *Rm. vannielii* swarmer cells held anaerobically in the dark. Cell free extract containing 600,000 c.p.m. was immunoprecipitated for each sample. The autoradiogram exposure was one week.

Fig. 4.4 Cell volume distribution profiles of samples removed from a synchronised population of *Rm. vannielii* swarmer cells after 0 hours, 2 hours, 4 hours and 6 hours anaerobic incubation in the dark.





The cell volume profiles in Fig. 4.4 show that under these conditions, the cell volume peak remains at the swarmer cell volume throughout the experiment. Light microscope observations confirmed that the swarmer cells failed to differentiate.

These results indicate (i) that expression of flagellin is developmentally regulated in *Em. vannielii*, and (ii) that the physiology and biochemistry of the bacterium enable it to sense the environment and decide whether or not to alter the expression of a specific gene accordingly.

The 2h-6h tracks in Fig. 4.1 act as controls to discount the idea that the flagellin immunoprecipitated in the 0h and 1h tracks in Fig. 4.1 and the 0h-6h tracks in Fig. 4.3 is synthesised by unsynchronised predivisional cells which are synthesising flagellin in order to assemble new flagella, as these tracks contain no immunoprecipitated labelled flagellin, yet the samples contain the same small proportion of unsynchronised cells as other samples.

It has been shown by radioimmunoprecipitation in *Caulobacter crescentus* (Laganaur and Agabian, 1978) and with the use of *hag-lac* fusions in *E. coli* (Komeda and Iino, 1979) that the flagellin gene is transcribed throughout the period when the bacterium is flagellate; in fact the flagellin gene is expressed constitutively in motile *E. coli* cells. Why flagellin continues to be made after flagella have been assembled is not known, but it is possible that

the tips of filaments become sheared during swimming, and must be constantly replaced.

It is not unlikely that control of flagellin expression in *Rm. vannielii* occurs at the level of transcription, as in *Caulobacter crescentus* (section 1.5.2.4). The expression of large numbers of genes must be controlled during the differentiation of a swarmer cell into a reproductive cell, which is the process studied using these experimental conditions. Moreover, in nature, when the mother cell produces a daughter cell by budding, the daughter cell has three possible fates: swarmer cell, reproductive chain cell or exospore, depending upon the prevailing environmental conditions (Fig. 1.13). The environmental conditions resulting in formation of each of the three cell types can be mimicked in the laboratory, therefore the study of the molecular biology of the control processes is possible.

Osley *et al.* (1977, section 1.5.1) found that transcription was not required for flagellin A synthesis in *Caulobacter crescentus* swarmer cells, and that flagellin B was not synthesised in swarmer cells. They documented the half life of flagellin A mRNA as 6.5 minutes, and that of flagellin B mRNA as 2.5 minutes. From these results they concluded that the flagellin A made in swarmer cells was translated from mRNA

transcribed before cell division. Results of dot blots performed by Milhausen and Agabian (1983, section 1.5.3) confirmed that flagellin mRNA synthesised in the predivisional cell is segregated with progeny swarmer cells at cell division. It was also observed that flagellin A synthesis in the swarmer cell decreased with a half life of 4 minutes (Milhausen and Agabian, 1983), which is similar to the previously recorded half life for flagellin A mRNA. Together, these findings led to the proposal that flagellin mRNA is not synthesised in the swarmer cell, and that switching off of flagellin synthesis in the differentiating swarmer cell is the result of the degradation of mRNA synthesised in the predivisional cell. Osley *et al.* (1977) suggested that if flagellin mRNA synthesised in the predivisional cell was segregated into the swarmer upon cell division, the longer functional half life of flagellin A mRNA would explain why flagellin A synthesis was detected in swarmer cells in the absence of transcription, whereas synthesis of flagellin B was not detected, due to flagellin B mRNA having a shorter half life.

From Fig. 4.3 it can be seen that, in *Rm. vannielii*, flagellin was synthesised in swarmer cells held anaerobically in the dark for at least six hours. This indicates that degradation of flagellin mRNA synthesised in the predivisional cell and segregated to the progeny swarmer cell is not the sole way in which flagellin synthesis is controlled in *Rm. vannielii* swarmer cells. If environmental conditions are not conducive to reproduction, *Rm. vannielii* swarmer cells have a means of controlling gene expression such that the flagellin gene or genes continue to be transcribed. It is not unlikely that *C. crescentus* can regulate its gene expression in a similar way, but because energy source and carbon source cannot be separated for *C. crescentus*, it is difficult to perform experiments allowing observation of such a

regulatory mechanism. However, as mentioned in section 1.6.1, there is evidence that *C. crescentus* swarmer cells accumulate when late exponential phase is reached (Swoboda, 1979), which suggests that these swarmer cells also fail to initiate differentiation unless environmental conditions are conducive to completion of the cell cycle.

Scott and Dow (1986b) investigated whether mRNA was synthesised in *Rm. vannielii* swarmer cells held anaerobically in the dark. They found that *de novo* RNA synthesis is required for the maintenance of protein synthesis in non-differentiating swarmer cells. This is in agreement with the conclusion drawn from the information in Fig. 4.3, but, to be specific about flagellin mRNA synthesis in non-differentiating *Rm. vannielii* swarmer cells, a cloned flagellin gene (or genes) is required as a probe.

It has been suggested by Porter and Dow (1987) that diminished intracellular ATP levels may mediate in the sensing of low light intensity by *Rm. vannielii*.

Low light intensity favours swarmer cell production (Dow *et al.*, 1983), which is useful when synchronising swarmer cells from a late exponential phase batch culture, as the self shading which occurs as the culture optical density increases results in an increased yield of swarmer cells. Interestingly Komeda and Iino (1979) found that expression of the flagellin gene in *E. coli* increased as logarithmic growth progressed. They concluded from this that flagellin gene expression must be controlled by some cellular metabolite in *E. coli*, and proposed cAMP as a possible candidate.

Light is known to be involved in differentiation in other bacteria. In

certain filamentous cyanobacteria akinetes form (see section 1.4.2) after cultures enter non-exponential growth due to light limitation (Dow *et al.*, 1983), and there is evidence that light is the factor which induces germination of akinetes (Dow *et al.*, 1983). Also, when the myxobacterium *Stigmatella aurantiaca* is starved on an agar surface, light is required at the stage of aggregate formation for the development of fruiting bodies (Qualls *et al.*, 1978).

To summarise, these experiments have identified that synthesis of flagellin is regulated during the cell cycle of *Rm. vannielii*, its period of synthesis coinciding with the possession of flagella by the cell, as is the case with *Caulobacter crescentus*. They also show, however, that flagellin synthesis continues indefinitely when environmental conditions are not conducive to completion of the cell cycle.

## CHAPTER 5

## ATTEMPTED CLONING OF FLAGELLIN GENE SEQUENCES

## 5 ATTEMPTED CLONING OF FLAGELLIN GENE SEQUENCES

Attempts were made to clone the flagellin gene (or genes) from *Rm. vannielii* in order to use this developmentally regulated gene as a probe in the study of control of flagellin expression and localisation.

Several examples of the types of experiment that can be done (and have been done with *Caulobacter* and *Bacillus*), using cloned genes that are regulated during differentiation, are described in the introduction of this thesis (sections 1.5.2, 1.5.3 and 1.4.1). They have yielded information on when the gene is transcribed, how the positioning of the gene product is controlled, the dependence of expression of one gene on expression of another, whether the chromosomal location of a gene is important for its timed expression and the role of 5' regulatory sequences.

Approaches used to clone the *Rm. vannielii* flagellin gene or genes were (i) DNA hybridisation using a cloned *C. crescentus* flagellin gene as a probe, (ii) use of the expression vector  $\lambda$ gt11 with anti-flagella antiserum as a probe and (iii) isolation of Tn5-induced motility mutants.

When this study was completed the flagellin gene had not been cloned, but the experience gained from the work done so far may be useful, and progress towards cloning the gene of interest was made. These are described below.

### 5.1 Hybridisations

The plasmid pFb1 (Fig. 5.1) was a kind gift from Dr. L. Shapiro. It is pBR325 carrying a 2.1 kb *Sall* insert containing the gene for the 29k flagellin from *Caulobacter crescentus* (Loewy *et al.*, 1987).

pFb1 was transformed into *E. coli* DH1, and a large scale plasmid preparation was carried out as described in section 2.28.2. Purified plasmid DNA was digested with *Sall*, and the resulting fragments were separated in a low melting temperature agarose gel. The 2.1 kb fragment was isolated from the gel as described in section 2.37, and could be labelled with [ $\alpha$ <sup>32</sup>P]dCTP (section 2.38.1) and used as a probe in Southern hybridisation.

Fig. 5.2 shows the result obtained when samples of *Rm. vannielii* chromosomal DNA were digested with three restriction enzymes and the fragments separated in an agarose gel, denatured and transferred to nitrocellulose (section 2.38.2). Hybridisation with the labelled, denatured *C. crescentus* flagellin probe was allowed to proceed overnight at 55°C in hybridisation fluid containing 0.75 M NaCl (5 x SSPE, section 2.38.3).

The hybridisation stringency can be varied by varying the conditions of temperature and salt concentration of the hybridisation reaction. When the probe DNA and the test DNA are heterologous, as in this case, it is useful to perform the hybridisation at a very low stringency (e.g. the conditions described above), and wash at increasing stringencies, analysing the result of each wash (Meinkoth and Wahl, 1984).



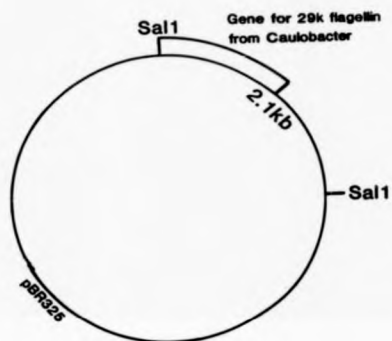


Fig. 5.1 pFB1

Below is a list of the wash stringencies used to produce the autoradiograms in Fig. 5.2. The percentage of homology, and the converse percentage of mismatch, between the probe DNA and any *Rm. vannielii* DNA band it lit up if it washed off at a particular stringency are given.

Fig. 5.2

	Wash conditions	% homology if probe washed off in these conditions	% mismatch if probe washed off in these conditions
(a)	55°C, 5 x SSC (0.75 M NaCl)	49	51
(b)	55°C, 2 x SSC (0.3 M NaCl)	56	44
(c)	55°C, 1 x SSC (0.15 M NaCl)	61	39

Conditions become less conducive to duplex formation, i.e. more stringent, as the temperature is increased and the salt concentration is decreased, and the converse is true.

The average base composition of the DNA used in hybridisations is also relevant to how likely duplex formation is under certain conditions. The average base composition of *C. crescentus* chromosomal DNA is 67 mol % G + C (Shapiro, 1976), and that for *Rm. vannielii* is 62 mol % G + C (Potts *et al.*, 1980). The mean of the two is 64.5 mol % G + C, and this value was used in calculations to obtain an estimate of sequence relatedness from the stringency of wash which abolished a particular duplex. This was done using the following equation:

$$T_m = 81.5^\circ\text{C} + 16.6 \log[\text{NaCl}] + 0.41 (\text{mol } \% \text{ G} + \text{C})$$

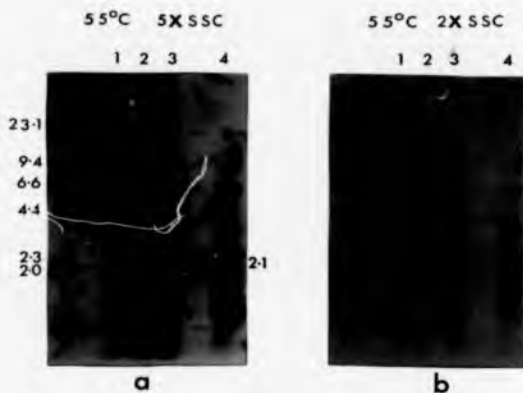
Fig. 5.2 Sequential washes of a Southern hybridization of the 29k flagellin gene probe from *C. crescentus* to *Rm. vannielii* genomic digests. The tracks contain:

1. Approximately 4  $\mu$ g of *Rhodocrobium vannielii* DNA digested with *EcoRI*.
2. Approximately 4  $\mu$ g of *Rhodocrobium vannielii* DNA digested with *cHindIII*.
3. Approximately 4  $\mu$ g of *Rhodocrobium vannielii* DNA digested with *Sall*.
4. Approximately 0.4  $\mu$ g of *Caulobacter crescentus* DNA digested with *Sall*.

The filter was exposed for 3 days for all 3 autoradiograms.

Size(kb)

170



In order to calculate percentage homologies it is also necessary to know that the  $T_m$  (melting temperature) of a DNA duplex decreases by  $1^\circ\text{C}$  with every 1% of base pairs which are mismatched (Meinkoth and Wahl, 1984).

Fig. 5.2(a) shows the autoradiogram of the filter after the first very low stringency wash. The positive control track containing *C. crescentus* chromosomal DNA digested with *Sall* shows that a 2.1 kb band does light up, along with several other bands containing flagellin gene sequences.

Fig. 5.2(b) shows that most of the probe DNA has washed off the *Rm. vannielii* DNA at a stringency which separates duplicates if they are less than only 56% homologous.

Fig. 5.2(c) shows the result of washing the filter under conditions which separate duplicates if probe and test DNA are less than 61% homologous. This is still not a high stringency wash, but almost all the *C. crescentus* probe DNA has washed off the test DNA, and there were no bands which showed strong hybridisation to a fragment of *Rm. vannielii* DNA. Very faintly hybridising bands are visible in the *EcoRI* track, corresponding to a 3.6 kb fragment from *Rm. vannielii* DNA, and in the *Sall* track, corresponding to a 3.8 kb fragment. However, these were not homologous enough to the *C. crescentus* probe to give a signal nearly strong enough to identify a clone from a *Rm. vannielii* gene bank by hybridisation.

Chromosomal DNA was prepared from some other members of the *Rhodospirillaceae* and probed with the *C. crescentus* flagellin probe, in the hope that a flagellin gene from one of these bacteria would be more

homologous to *C. crescentus* flagellin DNA. The thinking was that if the *C. crescentus* probe could be used to clone a flagellin gene from a bacterium in the same family as *Rm. vannielii*, this sequence could be used in turn to clone a flagellin gene from *Rm. vannielii*, i.e. the experiment was performed in an effort to find a flagellin sequence that showed some homology to both *C. crescentus* and *Rm. vannielii* DNA.

The results are shown in Fig. 5.3. Fig. 5.3(a) shows the autoradiogram of the filter washed at very low stringency. The contents of the tracks are given in the figure legend. All the DNAs were digested with *EcoRI*, with the exception of the *C. crescentus* control, which was again digested with *Sall*. *Rhodospseudomonas blautica* DNA was included as a negative control.

When the filter was washed at the same stringency as in Fig. 5.2(c), i.e. such that any duplex with homology of 61% or less would be denatured, the autoradiogram shown in Fig. 5.3(b) was obtained. Again the probe DNA has washed off the test DNA at low stringency, leaving no strongly hybridising bands, so the proposed intermediate step to cloning *Rm. vannielii* flagellin DNA by hybridisation could not be used.

These results (shown in Fig. 5.2) were disappointing in light of the fact that *C. crescentus* flagellin cross-reacted with antiserum raised against *Rm. vannielii* flagella (Fig. 3.8). This had engendered hope that there would also be sufficient homology at the DNA level to enable cloning by hybridisation. That this was not the case, despite the antibody homology, may have been because any regions of DNA homology were too short to be detectable by Southern hybridisation, or a result of the degeneracy of the genetic code.

Fig. 5.3 Sequential washes of a Southern hybridisation of the *C. crescentus* 29k flagellin probe to genomic digests of DNA from various members of the *Rhodospirillaceae*. The tracks contain:

1. Approximately 4  $\mu$ g *Rhodobacter capsulatus* DNA digested with *EcoRI*.
2. Approximately 4  $\mu$ g *Rhodopseudomonas viridis* DNA digested with *EcoRI*.
3. Approximately 4  $\mu$ g *Rhodopseudomonas palustris* DNA digested with *EcoRI*.
4. Approximately 4  $\mu$ g *Rhodocyclus gelatinosus* DNA digested with *EcoRI*.
5. Approximately 4  $\mu$ g *Rhodopseudomonas blastica* DNA digested with *EcoRI*.
6. Approximately 4  $\mu$ g *Rhodospirillum rubrum* DNA digested with *EcoRI*.
7. Approximately 4  $\mu$ g *Rhodomicrobium vannielii* DNA digested with *EcoRI*.
8. Approximately 0.4  $\mu$ g *Caulobacter crescentus* DNA digested with *Sall*.

Both autoradiograms were the result of a 3 day exposure.

Size(kb)

55°C 2X SSC

1 2 3 4 5 6 7 8



a

55°C 1X SSC

1 2 3 4 5 6 7 8



b

3.6

2.1



In Fig. 5.3(b), the weakly hybridising 3.6 kb *EcoRI* fragment from *Rm. vannielii* DNA is again visible. The *C. crescentus* flagellin gene hybridises slightly more strongly to this DNA than to any of the other *Rhodospirillaceae* DNA fragments, which could be taken as being in agreement with the antibody data, where antiserum raised against *Rm. vannielii* flagella reacted more strongly with a *C. crescentus* polypeptide than with proteins from the species of the *Rhodospirillaceae* tested.

#### 5.1.1 DNA sequence comparisons among bacterial flagellins

Homology among bacterial flagellins at the amino acid sequence level has been discussed in section 3.3. After obtaining the Southern hybridisation results described in the preceding section an examination of the relatedness of bacterial flagellins at the level of DNA sequence was carried out.

The European Molecular Biology Laboratory (EMBL) database was searched and was found to contain four bacterial flagellin DNA sequences. These are described in Table 5.1.

Fig. 5.4 shows the homologies which the computer found when asked to look for regions of 50% homology or more. These are described in the figure legend.

The N-terminal regions of flagellins are by far the most conserved regions of flagellin proteins and genes. Wei and Joys (1985) sequenced the genes for phase-1 flagellins from three species of *Salmonella*. Even from flagellins within one genus, the DNA sequence homology in the centre of the protein coding region was of the order of only 25%.

Table 5.1 Bacterial flagellin DNA sequences from EMBL database

Gene	No. of base pairs of sequence in database	Protein coding sequence in database	Reference
<i>Escherichia coli</i> hag	351 bp	60 bp of N-terminus	Szekely and Simon (1983)
<i>Salmonella</i> <i>typhimurium</i> H1	349 bp	60 bp of N-terminus	" "
<i>Salmonella</i> <i>typhimurium</i> H2	1149 bp	114 bp of N-terminus	" "
<i>Caulobacter</i> <i>croscensus</i> figJ	1193 bp	Whole protein	Gill and Agabian (1983)

Fig. 5.4 DNA sequence homologies of 50% or more among bacterial flagellin sequences in the EMBL database.

- (a) *Caulobacter crescentus* flgJ and *Escherichia coli* hag:  
60% homology over the first 60 bp of the protein coding regions.
- (b) *Caulobacter crescentus* flgJ and *Salmonella typhimurium* H2: 53% homology over the first 78 bp of the coding regions.
- (c) *Escherichia coli* hag and *Salmonella typhimurium* H2: 90% homology over the first 60 bp of the protein coding regions.
- (d) *Salmonella typhimurium* H1 and *Escherichia coli* hag: 83% homology over the first 60 bp of the protein coding regions.

200 210 220 230 240 250  
**figJ** GAAATC8CC GATCGGAGBT C8CAT8C8C TTAGC8TCAA CAC8AACCA8 CCC8C8CT8A  
 \* \* \* \* \*  
**hag** CTT8CAATAT AG8ATAAC8A ATCAT8C8C A8GTATTAA TACCAAC8C8 CTCTC8CT8A  
 269 279 289 299 309 319  
 260 270 280 290 300 310  
 TC8C8CT8CA GAATCTCAAC C8C  
 \* \* \* \* \*  
 TCACTCAAAA TAATATCAAC A88  
 329 339 349 359 369 379

a

200 210 220 230 240 250  
**figJ** GAAATC8CC GATCGGAGBT C8CAT8C8C TTAGC8TCAA CAC8AACCA8 CCC8C8CT8A  
 \* \* \* \* \*  
**H2** AAACCTTCCA A88888888 TTTAT8C8C A8GTATTAA CACTA8C8T CT8T8C8T8A  
 1013 1023 1033 1043 1053 1063  
 269 279 289 299 309 319  
 TC8C8CT8CA GAATCTCAAC C8C8CA8C8 A8GACAT8CA 8  
 \* \* \* \* \*  
 TGACCC88AA TAACCT88AC AAATCC88T C8C8CT888 C  
 1073 1083 1093 1103 1113 1123

b

250 260 270 280 290 300  
**hag** AGCCCAATAA CATCA8IT8 TAATT8AT8A 88888888C AT88C8CA88 TCATTATAT8  
 \* \* \* \* \*  
**H2** ATACAT888 T8ATCA888A CCTTCC888A 88888888T AT88C8CA88 TAATCA88C  
 996 1006 1016 1026 1036 1046  
 310 320 330 340 350 360  
 AAAC88C8T8 TC8CT8T8T8A C8C888AT8A CCT888CA8A  
 \* \* \* \* \*  
 TAAC88T8T8 TC8CT8C8T8A C8C888AT8A CCT888CA8A  
 1056 1066 1076 1086 1096 1106

c

270 280 290 300 310 320  
**H1** TAATT8AT8A 88888888C AT88C8CA88 TCATTATAT8 AAAC88C8T8 TC8CT8T8T8A  
 \* \* \* \* \*  
**hag** GCAATAT88 ATA8C8AT8C AT88C8CA88 TCATTATAT8 CAAC88C8T8 TC8CT8AT8A  
 272 282 292 302 312 322  
 330 340 350 360 370 380  
 C8C888AT8A CCT888CA8A  
 \* \* \* \* \*  
 CTC888AT8A TATCA8CA88  
 332 342 352 362 372 382

d

whereas the homology at the N-terminus protein coding region was 100%. The entire *E. coli* *hag* DNA sequence has also now been published by Kawajima et al., (1986), but has not yet been loaded on to the EMBL database. They compared this sequence with DNA sequences of genes encoding the phase-1 flagellin of *Salmonella typhimurium* and found only 20% homology in the central region, but 80% homology in the NH<sub>2</sub>-terminal region and 60% homology in the COOH-terminal region.

Only the first 60-100 bases of protein coding DNA of the *E. coli* and *S. typhimurium* flagellin genes were available from the EMBL database (Table 5.1), and the percentage homologies in the legend to Fig. 5.4 are high compared to the percentage homologies which would be obtained if the entire gene sequences were available. However, the equation described in section 5.1 can be applied to hybrids of 50 bp or greater (Meinkoth and Wahl, 1984), so 60 bp of homology is enough to identify DNA sequences by hybridisation.

Although the percentage homologies over 60 bp of N-terminus protein coding DNA from the related enteric bacteria *E. coli* and *S. typhimurium* are of the order of 83-90%, the homologies over comparable regions of DNA from *C. crescentus* and *E. coli* or *S. typhimurium* are of the order of 53-60%. This figure is similar to the homology found experimentally between *C. crescentus* flagellin DNA and *Em. vaniellii* DNA in this study. Together these observations led to the conclusion that heterologous DNA hybridisation is not a good method of detecting cloned flagellin genes.

## 5.2 Use of Antiserum in Cloning

A library of *EcoRI* digested *Rm. vannielii* chromosomal DNA was constructed in the vector  $\lambda$ gt11 (Fig. 5.5. and Young and Davis, 1983).

Purified  $\lambda$ gt11 DNA, which had been digested with *EcoRI*, which cuts it at a unique site, and had the resulting ends treated with alkaline phosphatase to give improved yields of recombinant phage was obtained from Promega Biotec. 0.5  $\mu$ g of  $\lambda$ gt11 DNA and 0.35  $\mu$ g of *EcoRI* digested *Rm. vannielii* DNA were ligated as described in section 2.33, and the DNA was packaged using a lambda *in vitro* packaging kit (Amersham International p.l.c.). This was plated and screened for *Rm. vannielii* flagellin production with antiserum 4 (section 3.1) as described in sections 2.39 and 2.41.

The library contained 40% recombinant phage, estimated from the percentage of phage in a sample from the library expressing  $\beta$ -galactosidase (section 2.40).  $2.9 \times 10^5$  phage were screened in total, thus  $1.2 \times 10^5$  phage containing an insert of *Rm. vannielii* DNA were screened, giving a very high probability that all the packageable *EcoRI* fragments in the *Rm. vannielii* genome were screened. The result was that no plaques bound anti-flagella antibodies.

The control shown in Fig. 5.6 indicates that the horseradish-peroxidase linked detection system for positive clones was operational. The filter on the left, (a), was overlaid on plaques produced by non-recombinant  $\lambda$ gt11 probed with anti- $\beta$ -galactosidase antiserum. The other filter, (b), was overlaid on plaques of wild type  $\lambda$  ( $\lambda$ C1<sub>85</sub>7<sub>8</sub>-7) and probed with anti- $\beta$ -galactosidase antiserum. Non-recombinant  $\lambda$ gt11 produces  $\beta$ -

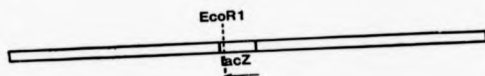


Fig. 3.5  $\lambda$ gt11 (modified from Young and Davis, 1983a).

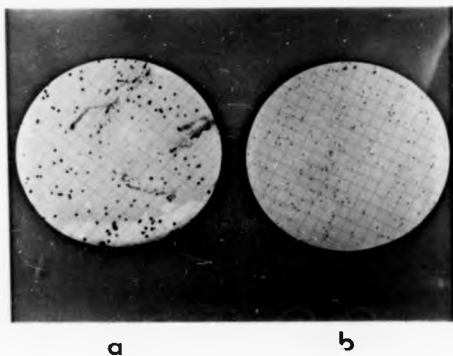


Fig. 5.6 Control for the horseradish peroxidase colour reaction used to detect positive  $\lambda$ gt11 clones. Filter (a) was overlaid on to  $\lambda$  plaques producing  $\beta$ -galactosidase, and probed with anti- $\beta$ -galactosidase antibody. Filter (b) was overlaid on to  $\lambda$  plaques not producing  $\beta$ -galactosidase and probed with anti- $\beta$ -galactosidase antiserum.



galactosidase; wild type  $\lambda$  does not, and this is apparent from the probed filters in Fig. 5.6. The antiserum used was raised to *E. coli*  $\beta$ -galactosidase (Sigma) as described in section 2.22, and gave an antigen/antibody precipitation line when rocket immunoelectrophoresis was carried out with the commercial  $\beta$ -galactosidase loaded in the antigen wells. Therefore it can be concluded from this experiment that neither *Rm. vannielii* flagellin nor hook protein are expressed from an EcoRI fragment of *Rm. vannielii* chromosomal DNA in  $\lambda$ gt11 in an *E. coli* host (*E. coli* Y1090).

Milhausen *et al.* (1982) refer to numerous unsuccessful efforts to identify *C. crescentus* flagellin genes by analysing host *E. coli* cells for polypeptide production from cloned DNA fragments. Although the hook protein gene from *C. crescentus* was cloned using a method similar to that described in this section, the DNA fragments used to construct the library were of a more random nature (they were generated by partial digestion with a restriction enzyme with a 4 bp recognition site) and it is thought that the expression of the *C. crescentus* protein was dependent on promoters in the vector DNA (Ohta *et al.*, 1982). Further evidence that *C. crescentus* promoters are not recognised by *E. coli* RNA polymerase was obtained by Chen *et al.* (1986) using the cloned hook operon of *C. crescentus* complete with 5' regulatory region. However, the neomycin phosphotransferase gene of Tn5 is expressed in a wide range of bacteria, including *C. crescentus* (Ely and Croft, 1982) and *Rm. vannielii* (R. Breadon, personal communication and section 5.3).

The reason for choosing  $\lambda$ gt11 as a vector originally was based on the fact that the identification of a cloned DNA fragment using antibody is dependent on transcription and translation of the polypeptide encoded by

the gene of interest. The requirement for the foreign promoter and translation signals to be utilized in *E. coli* is an obstacle which can be removed if a completely random library of foreign DNA is constructed in  $\lambda$ gt11. The foreign chromosomal DNA must be sheared mechanically by repeated passage through a narrow gauge syringe needle or by sonication to allow the generation of a library in which foreign inserts are present in all translational frames in both orientations. The synthesis of foreign proteins will then be driven by the  $\lambda$ gt11 *lacZ* promoter, and there are a number of other skilfully engineered features of  $\lambda$ gt11 described in Young and Davis (1983), Young and Davis (1983a) and Young *et al.* (1985) which make it an extremely useful expression vector for the detection of foreign proteins synthesised from DNA cloned in *E. coli*.

Construction of such a library takes time and can be technically difficult, but is a viable method of cloning *Rm. vannielii* flagellin DNA sequences. During the course of this study, a  $\lambda$ gt11 library containing sonicated *Rm. vannielii* chromosomal DNA fragments of 2-8 kb in size was constructed as in Young *et al.* (1985), with the exceptions that (a) sonication (as described in section 2.11, but with only one 0.5 second burst) was found to be more useful for generating random fragments of DNA than repeated passages through a 25 gauge needle, and (b) excess linkers were removed by spermine precipitation (Hoopes and McClure, 1981). Controls were carried out to ensure that the methylation reaction to protect *EcoRI* sites within the *Rm. vannielii* DNA during *EcoRI* digestion after linker addition, and linker addition itself (Maniatis *et al.*, 1982), were working.

This library was packaged and screened in the same way as the library of

*EcoRI* cut *Rm. vannielii* DNA. However, only  $2.6 \times 10^4$  plaque forming units resulted from this, and the percentage of these which were recombinant was 28. Thus, only 7280 recombinant phage were screened, and no phage producing *Rm. vannielii* flagellin or hook were found.

Young *et al.* (1985) suggest that of the order of  $10^6$  independent recombinant phage should be screened to be fairly sure of isolating a clone producing the protein of interest from a completely random library accommodating DNA inserts with endpoints that occur at each base pair throughout the foreign genome in both orientations. In this case the reason that a positive clone was not isolated was probably that too small a number of recombinant phage was screened.

The recombination and packaging efficiencies may be increased by altering the ratio of vector DNA to *Rm. vannielii* DNA, or using a different method for the removal of excess *EcoRI* linkers, e.g. passage over a gel filtration column, agarose gel electrophoresis, or both (Young *et al.*, 1985).

### 5.3 Tn5 Mutagenesis

#### 5.3.1 Introduction

During the period of this study, it was discovered that the transposon Tn5, which carries a kanamycin resistance determinant, could transpose into the *Rm. vannielii* chromosome (R. L. Breaston, personal communication).

The cloning of prokaryotic genes has been greatly facilitated by the use

of antibiotic-resistance transposons, as insertion of the transposon may occur within a gene, resulting in loss of gene function, and the fragment of DNA where this insertion has occurred can be identified by virtue of its acquired antibiotic resistance. The strategy described in section 1.5.2.2 for the cloning of the *C. crescentus* flagellin gene cluster (Purucker *et al.*, 1982) can then be followed to clone the wild type gene.

The donor *E. coli* strain S17-1 carrying the vector pSUP2021 (Table 2.2) described by Simon *et al.* (1983) was used. The following features of these two components of this transfer system make it ideal for transposon mutagenesis of Gram negative bacteria not closely related to *E. coli*.

S17-1 has the transfer genes of the broad host range plasmid RP4 integrated into its chromosome, which enable it to use any Gram negative bacterium as a recipient for conjugative DNA transfer (Simon *et al.*, 1983). Also, S17-1 is kanamycin sensitive, and can be selected against when it is used as the donor in Tn5 mutagenesis.

The mobilisable, Tn5-carrying vector, pSUP2021, is shown in Fig. 3.7 and is a pBR325 derivative. The plasmid is mobilisable from S17-1 because it has been constructed such that it carries the recognition site for mobilisation (mob site) that is used by the RP4-encoded transfer functions. Secondly, in order to mutagenise the bacterial chromosome with a transposon, it is necessary to eliminate the vector used to introduce the transposon. This can be done by using a plasmid that is not maintained in the recipient bacterium, and Simon *et al.* (1983) hold that vectors derived from pBR325 are unable to replicate in bacteria

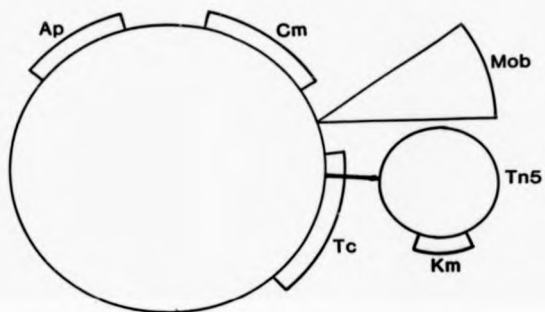


Fig. 5.7 pSUP2021 (from Simon *et al.*, 1983).

outside the enteric group.

### 5.3.2 Results of matings

Before any matings with *Rm. vannielii* were performed *E. coli* S17-1 carrying pSUP2021 was affirmed as being  $Ap^R Cm^R Km^R Tc^R$ , and a small scale plasmid preparation (section 2.28.3) was carried out to ensure that the plasmid was present as an extrachromosomal element. Finally, it was confirmed that S17-1 had the ability to transfer pSUP2021 to another *E. coli* strain by conjugation.

Eight matings were then carried out as described in section 2.42, using 10 ml of *Rm. vannielii* RE2 and 1 ml of *E. coli* S17-1 (pSUP2021) per mating, grown as described in section 2.42. *Rm. vannielii* strain RE2 is a nalidixic acid resistant derivative of strain Rm5, and is used here because it is necessary to select against donor cells (*E. coli* S17-1 (pSUP2021) is nalidixic acid sensitive). In all other experiments described in this thesis, the wild type strain, Rm5, of *Rm. vannielii* has been used.

After mating had proceeded overnight, the cells were washed off the filter with 2-4 ml of sterile PM medium. The suspension was concentrated to 1 ml by centrifugation, and 0.1 ml was spread on each of 10 PMY agar plates containing  $100 \mu g ml^{-1}$  of nalidixic acid to select against the *E. coli* donor,  $25 \mu g ml^{-1}$  of kanamycin to select for *Rm. vannielii* cells expressing the kanamycin resistance determinant encoded by *Tn5* and  $50 \mu g ml^{-1}$  of cycloheximide to prevent overgrowth by any contaminating fungi during the required 14 day incubation. The viable count of *Rm. vannielii* cells in the mating mixture was determined simultaneously by plating serial dilutions on PMY agar plates containing

100  $\mu\text{g ml}^{-1}$  nalidixic acid and 50  $\mu\text{g ml}^{-1}$  cycloheximide.

As a control, 10 ml of *Rm. vanielii* RB2 cells were filtered, incubated overnight and washed off the filter exactly as the mating cells had been, and plated on PMY agar plates containing 100  $\mu\text{g ml}^{-1}$  nalidixic acid, 25  $\mu\text{g ml}^{-1}$  kanamycin and 50  $\mu\text{g ml}^{-1}$  cycloheximide. Neat, 10x and  $10^2$ x dilutions on duplicate plates showed no growth after 14 days.

When the kanamycin plates containing the mating mixture were examined, it was found that there were several (on average approximately 12) large, diffuse colonies of *Rm. vanielii* per plate.

The frequencies of kanamycin resistant transconjugants were derived as follows:

	No. of kanamycin resistant colonies
Frequency of kanamycin	- <u>from a mating</u>
resistant transconjugants	No. of colony forming units of
	<i>Rm. vanielii</i> RB2 in the mating

The mean frequency of kanamycin resistant transconjugants obtained was  $1 \times 10^{-7}$ .

A total of 1062  $\text{Km}^r$  transconjugants was obtained, and these were patched on to three sets of PMY, 1.5% (w/v) agar plates, one containing 25  $\mu\text{g ml}^{-1}$  kanamycin, another containing 25  $\mu\text{g ml}^{-1}$  streptomycin and one containing 10  $\mu\text{g ml}^{-1}$  chloramphenicol. Finally, each  $\text{Km}^r$  transconjugant was stabbed into a motility assay plate, which contained PMY, 0.3% (w/v) agar and 25  $\mu\text{g ml}^{-1}$  kanamycin. Cells which are motile and

chemotactically competent will swarm in the sloppy agar, and those which are non-motile or defective in chemotaxis will fail to swarm, growing as a tight colony around the original stab (R. L. Breadon, personal communication). Fig. 5.8 shows such a plate, with examples of both colony types. Bryan *et al.* (1984) used similar conditions as an assay for chemotaxis in *C. crescentus*.

The transconjugants were patched on kanamycin to confirm that they were kanamycin resistant; on streptomycin as a backup to this, as Tn5 confers resistance to kanamycin and streptomycin on non-enterics; on chloramphenicol to assess whether pSUP2021 was eliminated; and on sloppy agar to identify motility and chemotaxis mutants. The results are in Table 5.2.

18 non-swarming transconjugants were initially isolated, but on restabbing into motility agar, only 13 were true non-swarming colonies. All were kanamycin resistant, all were also streptomycin resistant, and all were chloramphenicol sensitive.

Therefore, in this study,  $Ka^r$  transconjugants were obtained at a frequency of  $10^{-7}$ , and of these, 1.2% were defective in motility or chemotaxis.

In comparison, using the same vector in a similar donor mated with *Rhizobium meliloti*, Simon *et al.* (1983) obtained a frequency of  $10^{-4}$  neomycin resistant transconjugants, and Tn5-induced auxotrophic mutants were found with a frequency of 1-2%. Ely and Croft (1982) obtained a frequency of  $10^{-6}$  kanamycin resistant transconjugants using the vector pJB4JI to introduce Tn5 into *C. crescentus*. Approximately 1% of these



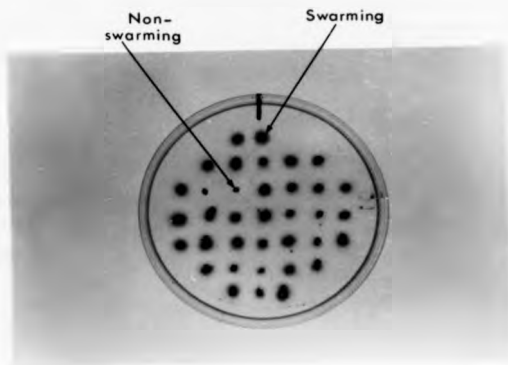


Fig. 5.8 An example of a swarm assay plate used to identify motility and chemotaxis mutants of *Rm. vannielii*. The agar concentration is 0.3% (w/v).

Table 5.2 Analysis of  $Km^r$  transconjugants from  
*E. coli* S17-1 (pSUP2021), *Rm. vannielii* RB2 mating

No. of $Sm^r$ colonies	Percentage $Sm^r$ colonies	No. of $Cm^r$ colonies	Percentage $Cm^r$ Colonies	No. of non- swarming colonies	Percentage non- swarming colonies
889	85	152	14	13	1.2

Km<sup>r</sup> cells were auxotrophic and approximately 2% had altered motility.

### 5.3.3 Tn5 induced, non-swarming mutants of *B. vanielii*

The 13 non-swarming colonies isolated were inoculated into liquid PMY containing 25  $\mu\text{g ml}^{-1}$  kanamycin. Many of them exhibited increased wall growth and clumping in comparison with wild type. All the mutants achieved a high culture optical density, with the exception of M10, which grew as clumps in the bottom of the flask, although it was grown in a shaking incubator.

The liquid cultures were examined in the light microscope for the presence or absence of motile cells. Care was taken to classify only those cells which were tumbling and swimming in different directions as swimmers, because single cells moved by liquid currents can look motile at first sight. Table 5.3 lists these results.

Non-swarming mutants can be divided into two groups by light microscopy. *fla* and *mot* mutants cannot swim, whereas *che* mutants are motile, but fail to form swarms in semisolid medium (Ely *et al.*, 1986). From Table 5.3, M1, M3, M9, M10, M13, M15 and M16 fall into the first group, and are *fla*<sup>-</sup> or *mot*<sup>-</sup>. M4, M17 and M18 are chemotaxis deficient.

It was also observed that cultures with a non-swarming phenotype often showed a marked reduction in the large multicellular arrays seen with wild type. Instead they showed a preponderance of single non-motile cells and unbranched chains of 2, 3 or 4 cells. Whittenbury and Dow (1977) state that no more than four daughter cells are ever formed by one mother cell. It appears that selecting for strains which do not produce swarmer cells selects for growth by unipolar extension without

Table 5.3 Light microscope observations of Tn5 induced  
non-swarmer mutants of *Rm. vannielii*

Mutant	Swarmer cells in light microscope	Check for swarming in motility agar
M1	-	-
M3	-	-
M4	+	-
M5	+	+/-
M6	-	-
M9	-	-
M10	-	-
M11	+	+/-
M13	-	-
M15	-	-
M16	-	-
M17	+	-
M18	+	-

branching. In the electron microscope it was seen that it was possible for these strains to form branches, but it was again apparent that far fewer large multicellular arrays were formed by strains which did not produce swimmers. Screening for non-swarming mutants of *Rm. vanneili* may pick up some mutants with non-lethal defects in development and growth as well as *fla*, *mot* and *che* mutants; M10 may be such a mutant.

It is possible to separate *fla* and *mot* mutations using the electron microscope. Those strains which cannot swim, but do have flagella are *mot*<sup>-</sup> and flagella-less strains are *fla*<sup>-</sup>. Paralysed (non wave-form) flagella and stub-forming mutants can also be identified in this way.

Preliminary electron microscopy showed that longer prosthecae than wild type were common among mutant cells. Long prosthecae are seen in wild type cultures when phosphate is limiting in the medium (Whittenbury and Dow, 1977). That prostheca length is increased in mutants may indicate that it is a general result of stress.

When *Rm. vanneili* RB2 or Ra5 is examined in the electron microscope, significant quantities of shed flagella can be seen on the grid. With the majority of the mutants classified as *fla*<sup>-</sup> or *mot*<sup>-</sup> shed flagella were not seen in the electron microscope. This suggests that swimmer cell flagella formation is not a necessary requirement for the *Rm. vanneili* cell cycle to proceed. This has also been found to be the case with *C. crescentus* (Shapiro, 1976). It also means that approximately six of the *fla* or *mot* group mutants may be *fla*<sup>-</sup>, and good candidates to clone the Tn5 insertion and corresponding wild type DNA from.

The fact that Tn5 will insert into the *Rm. vannielii* chromosome means that the use of the promoter probe Tn5-VB32 (Fig. 1.11) to assay transcription of genes with unknown or non-assayable protein products in *Rm. vannielii* is possible. This should prove very useful in future analyses of the control of expression of the flagellar genes in this photosynthetic bacterium.

Also, it is possible to clone the Tn5 insertions, with some flanking DNA, from the non-swarming mutants of *Rm. vannielii* using the selectable kanamycin resistance determinant. When these DNA fragments are labelled with  $^{32}\text{P}$  and used to probe a library of wild type *Rm. vannielii* DNA fragments, a number of regions of the *Rm. vannielii* chromosome encoding functions concerned with motility should be isolated.

In conclusion, cloning the Tn5 insertion from Tn5-induced *fla* mutants, constructing and screening a random  $\lambda$ gt11 expression library containing of the order of  $10^6$  recombinant phage or determining the amino acid sequence of the  $\text{NH}_2$ -terminus of the protein and constructing an oligonucleotide probe appear to be among the most promising approaches to cloning *Rm. vannielii* DNA sequences encoding flagellin.

## CHAPTER 6

## CONCLUDING REMARKS AND OUTLOOK

6 CONCLUDING REMARKS AND OUTLOOK

There is now a large body of evidence, from observations of the differing permeability of the two cell types, that the envelope structure of the *Rm. vannielii* swarmer cell differs from that of the reproductive cell (sections 1.6.2.3 and 3.4). The two cell types are functionally different; the envelope structure of the swarmer being related to its role as a quiescent, motile cell with a dispersal function, and the envelope structure of the reproductive cell being that of a growing and dividing cell.

From this it can be deduced that a change in envelope structure must take place during swarmer cell differentiation. This must include restructuring of the cell wall after flagella shedding, and preliminary work showed that a marked uptake of the peptidoglycan constituent diaminopimelic acid occurred after the loss of motility (C. S. Dow, unpublished). It is likely that membrane proteins required for chemotaxis and other taxes are present in the swarmer cell envelope and absent from the non-motile reproductive cell envelope, and this reorganisation may also occur after flagella shedding.

As pointed out by Macnab and Aizawa (1984), in all flagellated bacteria there must be a mechanism to prevent the extrusion of flagellin subunits from the cytoplasm to the distal tip of the flagellum constituting an ion leak. They suggest that there may be a special structure at the departure port on the cytoplasmic side of the flagellar basal body which allows the extrusion of flagellin monomers without allowing cell contents to leak out. Something of this nature must come into operation when flagella are shed from *Rm. vannielii* and *C. crescentus* cells.



It is likely that the apparatus for sensory transduction and chemotaxis is also involved in the change in envelope structure between swarmer cell and non-motile reproductive cell. Of course, how *Rm. vannielii* and other members of the *Rhodospirillaceae* carry out chemotaxis is of much interest in its own right, and it will be interesting, if the mechanism is elucidated, to relate it to the natural environment of these bacteria, which is typically a freshwater lake with nutrients present in low concentrations.

If the structure of basal bodies from *Rm. vannielii* was revealed, examination of the number of rings present would be possible. If they have five, like *C. crescentus* (Johnson *et al.*, 1979), this would lend weight to the hypothesis that the functional significance of having a flagellar basal body with five rings pertains to the shedding of flagella during the cell cycle of the bacterium.

Using synchronised swarmer cell populations, it has been found that flagellin synthesis in *Rm. vannielii* swarmer cells is switched off after anaerobic incubation in the light for 1-2 hours. However, this does not occur when swarmer cells are held anaerobically in the dark (Chapter 4). In order to gain more insight into the control processes involved, it would be useful to establish whether other proteins required for motility are coordinately regulated. This could be achieved in part by raising anti-hook antiserum to determine the period of hook protein synthesis. Initiation of hook protein synthesis occurs before flagellin synthesis in other bacteria (section 1.2.1), i.e. it is further back in the regulatory cascade, therefore this would be a good marker of the commitment of a daughter cell to being a swarmer.

One approach to unravelling the control mechanisms which operate during differentiation of *Caulobacter crescentus*, *Bacillus subtilis*, *Myxococcus xanthus* and *Streptomyces coelicolor* (sections 1.4 and 1.5) has been to perform experiments involving cloned genes which are developmentally regulated during differentiation. Hitherto, only 16S rRNA genes have been cloned from *Rm. vannielii* (Oakley, 1986), but with the advent of Tn5 mutagenesis of *Rm. vannielii* (section 5.3) it should now be possible to build up a collection of cloned, developmentally regulated genes from this organism, which have various patterns of expression.

Overall, because large volumes of synchronised *Rm. vannielii* cells can be easily obtained, and differentiation can be controlled by altering the environment of the cells in the laboratory, it can be concluded that research on *Rm. vannielii* has much to offer the field of bacterial differentiation. In addition, this research has increased knowledge of the functional adaptation of photosynthetic bacteria to their environment and can do so further in the future.

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